TITLE: PLASMODIUM SP. CHITINASE

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PLASMODIUM SP. CHITINASE

This application claims priority of U.S. Provisional Patent Application No. 60/136,508, filed May 28, 1999,

5 and of U.S. Provisional Patent Application No. 60/180,051, filed February 3, 2000.

FIELD OF THE INVENTION

The present invention relates generally to a

10 parasite protein, and more particularly to Plasmodium sp. chitinase and uses thereof.

BACKGROUND OF THE INVENTION

Throughout this application various publications are

15 referenced, many in parenthesis. Full citations for each of these publications are provided at the end of the Detailed Description and throughout the Detailed Description. The disclosures of each of these publications in their entireties are hereby incorporated

20 by reference in this application.

Defining molecular targets for drug or vaccine intervention remains a key strategy for developing new ways to prevent and treat malaria, a disease that exacts an enormous social and economic toll worldwide. A number

25 of investigators have proposed transmission-blocking vaccines as one component of an overall program of malaria control (Kaslow 1997). Such vaccines are designed to induce antibodies in humans that, when ingested by the mosquito along with a

30 Plasmodium-containing blood meal, interfere with the development of the parasite within the mosquito midgut. Animal models of transmission-blocking vaccines, based primarily on two P. falciparum zygote/ookinete surface proteins, Pfs25 and Pfs28, have demonstrated proof of

35 principle (Gozar et al. 1998), but results of human clinical trials have not been reported to date.

A Plasmodium ookinete-secreted enzyme, chitinase
(E.C. 3.2.1.14), has been demonstrated to be another
target of blocking malaria transmission from humans to
mosquitoes (Shahabuddin et al. 1993). Chitinases are
5 found in prokaryotes and eukaryotes (Flach et al. 1992);
their biologic roles include cell wall modification (e.g.
fungi (Kuranda and Robbins 1991), Entamoebae (Willagomez-
Castro et al. 1992) and filaria parasites (Fuhrman and
Piessens 1985)), carbon source degradation (e.g.
10 Streptomyces spp. (Ni and Westpheling 1997; Robbins et
al. 1988), Serratia marcescens (Roberts and Cabib 1982),
and Vibrio spp. (Keyhani and Roseman 1996)), and plant
and fungal host defense against chitin-containing
pathogens (Flach et al. 1992). One other protozoan
15 pathogen of man, Leishmania donovani, the agent of human
visceral leishmaniasis, is known to use a chitinase in
its life cycle (Schlein et al. 1991; Shakarian and Dwyer
1998). The Leishmania chitinase is thought to disrupt
the sand fly cardiac valve, allowing amastigotes to be
20 regurgitated from the midgut into the skin of the
vertebrate host. The Leishmania chitinase is not thought
to function in invasion of the arthropod vector per se
(Schlein et al. 1992). In contrast, Plasmodium chitinase
is thought to be required for the parasite to invade the
25 mosquito midgut after being taken up in a blood meal
(Shahabuddin et al. 1993). Because of its critical
biological function in the life cycle of the malaria
parasite, the Plasmodium chitinase is a potential target
for blocking transmission from the vertebrate host to the
30 mosquito vector (Shahabuddin et al. 1993).

The potential importance of chitinase in malaria
parasite biology was first suggested by a transmission
electron micrograph showing the P. gallinaceum ookinete
penetrating and appearing to focally degrade the

chitinous peritrophic matrix (PM) in the Aedes aegypti
midgut (Sieber et al. 1991). The PMs of the Plasmodium
vectors Anopheles gambiae (which carries human malaria
parasites) and A. aegypti (which carries avian malaria

5 parasites) are composed of chitin, a β-1,4-linked polymer
of GlcNAc, with intercalated proteins including trypsins
and peritrophins (Perrone and Spielman 1988; Shen and
Jacobs-Lorena 1997; Shen and Jacobs-Lorena 1998). P.
gallinaceum ookinetes secrete active chitinase (Huber et

10 al. 1991; Vinetz and Kaslow 1998). Although chitinases
are found throughout the prokaryote and eukaryote
kingdoms, the biological function of Plasmodium
chitinases must be different, because ookinetes do not
contain chitin and there is no evidence that ookinetes

15 use chitin or mono- or oligomers of GlcNAc as a carbon
source. Chitinases are critical for allowing the
parasite to escape the mosquito midgut, as evidenced by
the observation that addition of the chitinase inhibitor
allosamidin to a blood meal prevents oocyst development

20 (Shahabuddin et al. 1993). Both P. gallinaceum in A.
aegypti and P. falciparum in A. freeborni fail to develop
into oocysts in the presence of this inhibitor. This
effect could be completely reversed by enzymatic
degradation of the peritrophic matris (PM) in vivo, by

25 adding exogenous chitinase to the blood meal. These
observations demonstrated that a chitinase is necessary
for malaria parasites to invade the mosquito and initiate
sporogonic development.

Because of intrinsic biologic interest and the
30 potential for Plasmodium chitinases to be targets of
interfering with malaria transmission (for a review of
malaria transmission-blocking vaccines and the potential
of Plasmodium chitinases as targets, see: Kaslow 1993;

Shahabuddin and Kaslow 1993), a need exists for the identification of the malarial parasite chitinase.

SUMMARY OF THE INVENTION

5   To this end, the subject invention provides an isolated nucleic acid molecule encoding a Plasmodium sp. chitinase. The invention also provides an oligonucleotide complementary to at least a portion of the mRNA encoding the Plasmodium sp. chitinase.

10   The isolated nucleic acid molecules of the invention can be inserted into suitable expression vectors and/or host cells. Expression of the nucleic acid molecules encoding the Plasmodium sp. chitinase results in production of Plasmodium sp. chitinase in a host cell.

15 Expression of the oligonucleotide in a host cell results in decreased expression of the Plasmodium sp. chitinase.

The invention further provides methods of screening a substance for the ability of the substance to modify Plasmodium sp. chitinase function, and a method of

20 obtaining DNA encoding a Plasmodium sp. chitinase.

Further provided is an isolated nucleic acid molecule encoding a Plasmodium sp. chitinase, wherein the nucleic acid molecule encodes a first amino acid sequence having at least 90% amino acid identity to a second amino

25 acid sequence. The second amino acid sequence is as shown in SEQ ID NO:3 or SEQ ID NO:4.

The invention further provides a DNA oligomer capable of hybridizing to a nucleic acid molecule encoding a Plasmodium sp. chitinase. The DNA oligomer

30 can be used in a method of detecting presence of a Plasmodium sp. chitinase in a sample, which method is also provided by the subject invention.

The invention also provides an isolated Plasmodium sp. chitinase, a composition thereof, and antibodies or

antibody fragments specific for the Plasmodium sp. chitinase. The antibodies and antibody fragments can be used to detect the presence of the Plasmodium sp. chitinase in samples. The subject invention further

5 provides a method of producing an antibody specific for a Plasmodium sp. chitinase in a host. The method comprises selecting the isolated Plasmodium sp. chitinase or an antigenic portion thereof and introducing the selected Plasmodium sp. chitinase or antigenic portion thereof

10 into a host to induce production of an antibody specific for Plasmodium sp. chitinase in the host. Further provided is an isolated Plasmodium sp. chitinase encoded by a first amino acid sequence having at least 90% amino acid identity to a second amino acid sequence, the second

15 amino acid sequence as shown in SEQ ID NO:3 or SEQ ID NO:4.

The subject invention further provides a method of preventing infection of mosquitoes by Plasmodium sp., the method comprising exposing the Plasmodium sp. to an

20 amount of a compound effective to interfere with function of Plasmodium sp. chitinase, thereby preventing infection of the mosquitoes by the Plasmodium sp.

Further provided is a method of preventing transmission of malaria by a mosquito feeding on a

25 subject that may harbor Plasmodium sp. organisms. The method comprises administering to the subject an amount of a composition of the Plasmodium sp. chitinase effective to induce production of an antibody specific for Plasmodium sp. chitinase in the subject, wherein the

30 antibody inhibits Plasmodium sp. chitinase and is transferred to a mosquito feeding on the subject thereby preventing infection of the mosquito by Plasmodium sp. organisms that may be harbored in the subject.

Alternatively, the method of preventing transmission of malaria by a mosquito feeding on a subject that may harbor Plasmodium sp. organisms can comprise administering to the subject an amount of a compound

5 effective to interfere with function of Plasmodium sp. chitinase in the subject, wherein the compound is transferred to a mosquito feeding on the subject thereby preventing infection of the mosquito by Plasmodium sp. organisms that may be harbored in the subject.

10 Also provided is a method of preventing transmission of malaria by a mosquito that ingests Plasmodium sp. organisms. The method comprises introducing into the mosquito an amount of a compound effective to interfere with function of Plasmodium sp. chitinase thereby

15 preventing infection of the mosquito by ingested Plasmodium sp. organisms.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features and advantages of this

20 invention will be evident from the following detailed description of preferred embodiments when read in conjunction with the accompanying drawings in which:

Figs. 1a-1e illustrate the purification of the P. gallinaceum chitinase. Fig. 1a: quaternary ammonium

25 anion exchange HPLC of crude ookinete extracts. The gradient from 20 to 500 mM NaCl was developed over 30 min. Chitinase-containing fractions (*) eluted at 150-250 mM NaCl. Fig. 1b: hydrophobic interaction HPLC. Chitinase-containing fractions from 1a were injected into

30 a phenyl-TSK hydrophobic interaction column. Chitinase-containing fractions eluted 18 min after the ammonium sulfate concentration reached 0 M. Fig. 1c: because of a high base line, the chitinase-containing fractions from 1b were re-injected onto the same

hydrophobic interaction column (HIC). Fig. 1d:
chitinase-containing fractions from 1c were injected into
a C18 reverse-phase HPLC column. Fig. 1e:
SDS-polyacrylamide gel electrophoresis and Coomassie Blue
5 staining of fractions from 1d. Solid lines in the panels
indicate absorbance; lines with ◆ indicate chitinase
activity.;

Fig. 2 illustrates the cloning strategy of PgCHT1
cDNA. Reverse transcriptase PCR was performed using as
10 template first strand cDNA (prepared with the Capfinder
system; see Materials and Methods) synthesized from P.
gallinaceum ookinete total RNA and degenerate
oligonucleotide primers based on the amino acid sequences
of GT29 and GT84, as indicated by arrowheads within
15 rectangles. The resulting PCR product was cloned and
sequenced, from which the non-degenerate PCR primers 2503
and 2501 were designed. Primer 2501 paired with the
Capfinder 5' oligonucleotide primer and Primer 2503
paired with the oligo(dT) primer (see Materials and
20 Methods for primer sequences) were used for two separate
PCR reactions using first strand ookinete cDNA as
template. A single product was obtained with the primer
pair 2501 × 5' PCR primer; this PCR product was cloned
and sequenced. Several discrete bands were obtained with
25 the primer pair 2503 × oligo(dT); only the largest of
these bands was cloned and sequenced. The sequences of
these two reverse transcriptase-PCR products overlapped
and gave rise to the full-length 2508-bp cDNA of PgCHT1
(GenBank™ accession number AF064079);

30 Fig. 3 shows the complete amino acid sequence of the
P. gallinaceum chitinase gene, PgCHT1. Dashed box
indicates putative signal sequence. N-terminal 1 and
N-terminal 2 indicate the amino termini determined by
direct Edman degradation sequencing of the purified

60-kDa doublet (Fig. 1e, fractions 62-64). GT29, GT33, and GT84 indicate the Edman degradation-determined amino acid sequences of tryptic peptides derived from the 60-kDa doublet. Barbells indicate the synthetic peptides

5 used for making antisera (see Materials and Methods). Short arrows indicate sites of proteolysis by Endo Lys-C. The consensus catalytic active site and substrate-binding site are labeled and shaded. * indicates the termination codon;

10 　　　Figs. 4a-4c show the different forms of P. gallinaceum ookinete-produced chitinase and time course of expression. Fig. 4a: aliquots of fractions 65 and 66 from Fig. 1e were subjected to 4-20% SDS-PAGE and transferred to nitrocellulose. The same blot was

15 sequentially reacted with the carboxyl terminus antiserum (C-term) (right panel), stripped, and then reacted with the active site (AS) antiserum (left panel). Figs. 4b and 4c show Western immunoblot and determination of enzymatic activity, performed in a time course

20 experiment. At 1.5 h after exflagellation and fertilization, the parasites are in the zygote stage. At 10 h, the intermediate form in which ookinetes are exiting the residual body, the retort stage, are present. Fully mature ookinetes are not seen until about 15 h

25 after zygote formation. Fig. 4b is a Western immunoblot of parasite proteins at various time points after zygote formation, using antisera directed against synthetic peptides derived from the catalytic active site and the carboxyl-terminal domain. Parasite proteins were

30 extracted with a mixture of protease inhibitors (see Materials and Methods). An equal number of parasite equivalents were loaded into each lane or assayed for chitinase activity. Single arrowheads indicate proteins associated with peak 1 of chitinase activity; double

arrowheads indicate proteins associated with peak 2 of chitinase activity. The thin arrow indicates the precursor of the protein doublet of 68-kDa indicated by the upper single arrowhead, as demonstrated by a Western

5   immunoblot using antisera directed against the pro-enzyme domain. Protein bands that do not increase in intensity during the course of ookinete development, and thus presumably are not chitinases, are not indicated with arrowheads. Fig. 4c shows chitinase activity, as

10   detected with 4-MU GlcNAc$_3$, as a function of time after zygote formation;

Figs. 5a-5e show two chromatographically separable chitinase activities produced by P. gallinaceum ookinetes. Fig. 5a shows that quaternary ammonium anion

15   exchange HPLC of ookinete extracts using a shallower gradient than that used in Fig. 1a was able to separate peaks of chitinase activity. Proteins from each peak of chitinase activity were subjected to SDS-PAGE with a 4-20% polyacrylamide gel under both non-reducing and

20   reducing conditions and immunoblotted (5b-5e). Figs. 5b and 5d show non-reduced and reduced, respectively, probed with active site antiserum; Figs. 5c and 5e show non-reduced and reduced, respectively, probed with carboxyl-terminal antiserum;

25   Figs. 6a-6d show the effect of Endo Lys-C on P. gallinaceum ookinete-produced chitinase. Concentrated ookinete supernatants (from ~2 × 10^7 parasites per lane) were either treated with Endo Lys-C or buffer alone. Aliquots were taken for immunoblotting at 5, 30, and 120

30   min. An equal volume of 2× sample buffer with or without 10% β-mercaptoethanol was added to an aliquot of each fraction, and the samples were subjected to SDS-PAGE with a 16% polyacrylamide gel. After electroblotting, the

blots were probed with active site or carboxyl terminus
antiserum;

Figs. 7a-7c show the expression of recombinant
PgCHT1-NT1 and demonstrates that it is enzymatically
5 active. Fig. 7a: the NT1 form of PgCHT1 (amino acids
(aa) Tyr^{65} to Gln^{587}) was amplified from a synthetic PgCHT1
gene constructed in E. coli preferred codons with NcoI
and XhoI restriction sites included in the 5' and 3' ends
of the PCR primers, respectively. The NcoI and XhoI
10 restriction enzyme-digested PCR product was cloned into
the NcoI and XhoI restriction sites of the bacterial
expression vector, pET32b. This vector expresses
proteins fused to a 105-amino acid thioredoxin (trx)
leader sequence and to hexahistidine tags (His$_6$) at both
15 the amino and carboxyl termini. An enterokinase cleavage
site allows for removal of the amino-terminal fusion
protein, leaving the correct NT1 amino terminus of
rPgCHT1-NT1. Endo Lys-C was experimentally determined
(see Figs. 6a-6d and text) to cleave rPgCHT1-NT1 as shown
20 schematically. The PgCHT1-NT1 construct used for
enzymological analysis corresponds to one of the forms of
the PgCHT1 gene product secreted by ookinetes, as
determined by direct amino-terminal sequencing of the
purified 60-kDa chitinase (Fig. 1e and Fig. 3). Fig. 7b:
25 rPgCHT1-NT1 degrades polymeric chitin in a glycol chitin
activity gel. Equal amounts of rPgCHT1-NT1 (prepared and
treated as described under Materials and Methods) were
electrophoresed in a native, non-denaturing 8%
polyacrylamide gel into which 0.02% glycol chitin had
30 been incorporated. After the gel was run and was
incubated in 0.1 M sodium phosphate, pH 6.8, the gel was
counter-stained with Calcofluor White and visualized by
transillumination with UV light. Fig. 7c: rPgCHT1-NT1
cleaves 4-MU GlcNAc$_3$ and is enhanced by Endo Lys-C but not

enterokinase proteolytic treatment. Chitinase activity
is represented as fold change of relative fluorescence
units;

Fig. 8 shows thin layer chromatography analysis of
5 native chitin oligosaccharide substrate preference of
recombinant PgCHT1 and reaction products resulting from
enzymatic hydrolysis. Substrate lengths $GlcNAc_{1-6}$ are
indicated on top of the photograph. Reaction products
are indicated on the right. The origins where the
10 reaction mixtures were spotted are as indicated;

Figs. 9a-9d show the analysis of activity of crude
P. gallinaceum ookinete extracts and rPgCHT1 on 4-MU
derivatives of chitin oligosaccharides. Fig. 9a: TLC
analysis of end products produced by P. gallinaceum
15 ookinete crude extracts on 4-MU $GlcNAc_{1-4}$ substrates.
Fig. 9b: quantitation of fluorescence produced by P.
gallinaceum ookinete crude extracts on 4-MU $GlcNAc_{1-4}$
substrates. Fig. 9c: TLC analysis of end products
produced by Endo Lys-C-treated rPgCHT1 on 4-MU $GlcNAc_{1-4}$
20 substrates. Fig. 9d: quantitation of fluorescence
produced by Endo Lys-C-treated rPgCHT1 on 4MU $GlcNAc_{1-4}$
substrates. Substrate lengths are indicated on top;
reaction products are indicated at right. S, at left of
panels 9a and 9c indicates unreacted substrates as
25 standards. Free 4-MU fluorescences 12 times that of 4-MU
derivatives of GlcNAc oligomers; thus, the weaker
fluorescent bands corresponding to the mono-, di- and
tetra-MU cannot be compared stoichiometrically to the
free 4-MU;

30 Figs 10a and 10b show the analysis of pH activity
profile and allosamidin sensitivity of the two peaks of
chitinase activity chromatographically separated from P.
gallinaceum ookinete extracts as in Figs. 6a-6d and Endo
Lys-C-activated rPgCHT1-NT1. Chitinase activity is

expressed as percent activity of enzyme in the absence of
inhibitor (as detected with the 4-MU GlcNAc$_3$ substrate).
---, peak 1 of chitinase activity; ●, rPgCHT1; ◊, peak 2
of chitinase activity. These experiments were repeated

5   two times each with two different preparations of enzyme;

    Fig. 11 illustrates a comparison of amino acid
sequences of PfCHT1 and PgCHT1. The predicted signal
peptides are underlined. Putative proenzyme, catalytic,
and chitin-binding domains are indicated;

10 substrate-binding and catalytic active sites are
overlined. NT1 and NT2 delineate the two secreted forms
of PgCHT1;

    Figs. 12a-12c illustrate restriction mapping,
chromosomal localization, and transcriptional activity of

15 the PfCHT1 gene. Fig. 12a is a Southern blot of P.
falciparum strain 3D7. Restriction enzymes used to
digest the DNA are shown across the top; molecular sizes
are indicated in bp at left. Fig. 12b shows chromosomal
localization of PfCHT1 by PCR on pulse-field

20 gel-electrophoresis-separated P. falciparum chromosomal
DNA. Gene-specific primers demonstrate that PfCHT1 is
located on chromosome 12. Std, 500-bp DNA ladder, shown
at left. Chromosome numbers are at the top. Dd2,
genomic DNA template from P. falciparum strain Dd2 used

25 as a positive control. Fig. 12c shows reverse
transcription-PCR (RT-PCR) analysis to determine the
presence of PfCHT1 message in total RNA extracted from P.
falciparum-infected A. freeborni midguts. The same
preparation of RNA was used for RT-PCR of both PfCHT1 and

30 Pfs28. RT-PCR of Pfs28 mRNA, which encodes an P.
falciparum zygote/ookinete surface protein, was included
as a positive control. The 100-bp ladder is indicated;
+RT and RT, with and without reverse transcriptase;

Figs. 13a-13c show the purification of enzymatically active rPfCHT1. In Fig. 13a, a lysate of induced bacteria cell mass was clarified, run over a nickel-Sepharose column, and eluted with a step gradient

5 of imidazole in 1 M NaCl/20 mM Tris, pH 8.0. Fractions were assayed for chitinase activity by microfluorimetry with 4MU-GlcNAc$_3$. In Fig. 13b, samples were analyzed by SDS/PAGE; the gel was stained with Coomassie blue. In Fig. 13c, treatment of pooled fractions 21 and 22 with

10 enterokinase (EK) after dialysis of the protein against 20 mM Tris, pH 7.5/50 mM NaCl/2 mM CaCl$_2$ is shown;

Figs. 14a-14c show analysis of the action of rPfCHT1 on native chitin oligosaccharide substrates and 4MU derivatives of chitin oligosaccharides. Fig. 14a shows

15 TLC analysis of end products produced by rPfCHT1 on native GlcNAc$_{1-6}$. Fig. 14b shows TLC analysis of end products produced by rPfCHT1 on 4MU-GlcNAc$_{1-4}$. Oligosaccharide substrate lengths are indicated at the top, and reaction products are shown at right. The

20 origins at which the reaction mixtures were spotted are as indicated. Free 4MU fluoresces 12 times more than 4MU derivatives of GlcNAc oligomers; thus, the weaker fluorescent bands corresponding to the mono-, di-, and tetra-MU cannot be compared stoichiometrically to the

25 free 4MU. The origins at which the reaction mixtures were spotted are indicated. S, 4-MU GlcNAc$_{1-4}$ standards. Fig. 14c shows microfluorimetry analysis of initial rates of fluorescence produced by rPfCHT1 on 4MU substrates, as a measure of relative initial reaction rates of chitinase

30 activity;

Figs. 15a-15b show the pH activity profile and pH-dependent inhibition of rPfCHT1 by allosamidin. Fig. 15a shows the relative rates of rPfCHT1 activity at different pH levels. Shaded bar indicates that at pH

4.5, initial enzyme activity is linear for 10 min at 37°C, but then slows and is irreversibly gone at 20 min. rPfCHT1 has no chitinase activity at pH 3.5 or 4.0. Data are displayed as the mean of three separate experiments; errors are 5-7%. Fig. 15b shows relative rates of allosamidin inhibition of rPfCHT1 activity at pH 5.0 (■) and pH 6.0 and 7.0 (◆). 4MU-GlcNAc$_3$ was used as substrate for both sets of experiments; and

Fig. 16 shows the homology model depicting the overlapping catalytic sites of PfCHT1, PgCHT1, and human chitotriosidase complexed with allosamidin. The three active sites are almost perfectly superimposable, with the exception of a novel pocket found in PgCHT1, seen at lower right. The models were built by using the structure of hevamine complexed with allosamidin as a template.

DETAILED DESCRIPTION OF THE INVENTION

The term "nucleic acid", as used herein, refers to either DNA or RNA. "Nucleic acid sequence" or "polynucleotide sequence" refers to a single- or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5' to the 3' end. It includes both self-replicating plasmids, infectious polymers of DNA or RNA, and nonfunctional DNA or RNA.

"Isolated" nucleic acid refers to nucleic acid which has been separated from an organism in a substantially purified form (i.e. substantially free of other substances originating from that organism), and to synthetic nucleic acid.

By a nucleic acid sequence "homologous to" or "complementary to", it is meant a nucleic acid that selectively hybridizes, duplexes or binds to DNA sequences encoding the protein or portions thereof when

the DNA sequences encoding the protein are present in a
genomic or cDNA library. A DNA sequence which is similar
or complementary to a target sequence can include
sequences which are shorter or longer than the target

5 sequence so long as they meet the functional test set
forth.

Typically, the hybridization is done in a Southern
blot protocol using a 0.2X SSC, 0.1% SDS, 65°C wash. The
term "SSC" refers to a citrate-saline solution of 0.15M

10 sodium chloride and 20 mM sodium citrate. Solutions are
often expressed as multiples or fractions of this
concentration. For example, 6X SSC refers to a solution
having a sodium chloride and sodium citrate concentration
of 6 times this amount or 0.9 M sodium chloride and 120

15 mM sodium citrate. 0.2X SSC refers to a solution 0.2
times the SSC concentration or 0.03M sodium chloride and
4 mM sodium citrate.

The phrase "nucleic acid molecule encoding" refers
to a nucleic acid molecule which directs the expression

20 of a specific protein or peptide. The nucleic acid
sequences include both the DNA strand sequence that is
transcribed into RNA and the RNA sequence that is
translated into protein or peptide. The nucleic acid
molecule includes both the full length nucleic acid

25 sequences as well as non-full length sequences derived
from the full length protein. It being further
understood that the sequence includes the degenerate
codons of the native sequence or sequences which may be
introduced to provide codon preference in a specific host

30 cell.

The term "located upstream" as used herein refers to
linkage of a promoter upstream from a nucleic acid (DNA)
sequence such that the promoter mediates transcription of
the nucleic acid (DNA) sequence.

The term "vector", refers to viral expression systems, autonomous self-replicating circular DNA (plasmids), and includes both expression and nonexpression plasmids. Where a recombinant

5 microorganism or cell is described as hosting an "expression vector," this includes both extrachromosomal circular DNA and DNA that has been incorporated into the host chromosome(s). Where a vector is being maintained by a host cell, the vector may either be stably

10 replicated by the cells during mitosis as an autonomous structure, or the vector may be incorporated within the host's genome.

The term "plasmid" refers to an autonomous circular DNA molecule capable of replication in a cell, and

15 includes both the expression and nonexpression types. Where a recombinant microorganism or cell is described as hosting an "expression plasmid", this includes latent viral DNA integrated into the host chromosome(s). Where a plasmid is being maintained by a host cell, the plasmid

20 is either being stably replicated by the cell during mitosis as an autonomous structure, or the plasmid is incorporated within the host's genome.

The phrase "heterologous protein" or "recombinantly produced heterologous protein" refers to a peptide or

25 protein of interest produced using cells that do not have an endogenous copy of DNA able to express the peptide or protein of interest. The cells produce the peptide or protein because they have been genetically altered by the introduction of the appropriate nucleic acid sequences.

30 The recombinant peptide or protein will not be found in association with peptides or proteins and other subcellular components normally associated with the cells producing the peptide or protein.

The following terms are used to describe the
sequence relationships between two or more nucleic acid
molecules or polynucleotides, or between two or more
amino acid sequences of peptides or proteins: "reference
5 sequence", "comparison window", "sequence identity",
"sequence homology", "percentage of sequence identity",
"percentage of sequence homology", "substantial
identity", and "substantial homology". A "reference
sequence" is a defined sequence used as a basis for a
10 sequence comparison; a reference sequence may be a subset
of a larger sequence, for example, as a segment of a
full-length cDNA or gene sequence given in a sequence
listing or may comprise a complete cDNA or gene sequence.
    Optimal alignment of sequences for aligning a
15 comparison window may be conducted, for example, by the
local homology algorithm of Smith and Waterman (1981), by
the homology alignment algorithm of Needleman and Wunsch
(1970), by the search for similarity method of Pearson
and Lipman (1988), or by computerized implementations of
20 these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the
Wisconsin Genetics Software Package Release 7.0, Genetics
Computer Group, 575 Science Dr., Madison, Wis.).
    As applied to nucleic acid molecules or
polynucleotides, the terms "substantial identity" or
25 "substantial sequence identity" mean that two nucleic
acid sequences, when optimally aligned (see above), share
at least 90 percent sequence identity, preferably at
least 95 percent sequence identity, more preferably at
least 96, 97, 98 or 99 percent sequence identity.
30   "Percentage nucleotide (or nucleic acid) identity"
or "percentage nucleotide (or nucleic acid) sequence
identity" refers to a comparison of the nucleotides of
two nucleic acid molecules which, when optimally aligned,
have approximately the designated percentage of the same

nucleotides. For example, "95% nucleotide identity"
refers to a comparison of the nucleotides of two nucleic
acid molecules which when optimally aligned have 95%
nucleotide identity. Preferably, nucleotide positions
5 which are not identical differ by redundant nucleotide
substitutions (the nucleotide substitution does not
change the amino acid encoded by the particular codon).

As further applied to nucleic acid molecules or
polynucleotides, the terms "substantial homology" or
10 "substantial sequence homology" mean that two nucleic
acid sequences, when optimally aligned (see above), share
at least 90 percent sequence homology, preferably at
least 95 percent sequence homology, more preferably at
least 96, 97, 98 or 99 percent sequence homology.

15 "Percentage nucleotide (or nucleic acid) homology"
or "percentage nucleotide (or nucleic acid) sequence
homology" refers to a comparison of the nucleotides of
two nucleic acid molecules which, when optimally aligned,
have approximately the designated percentage of the same
20 nucleotides or nucleotides which are not identical but
differ by redundant nucleotide substitutions (the
nucleotide substitution does not change the amino acid
encoded by the particular codon). For example, "95%
nucleotide homology" refers to a comparison of the
25 nucleotides of two nucleic acid molecules which when
optimally aligned have 95% nucleotide homology.

As applied to polypeptides, the terms "substantial
identity" or "substantial sequence identity" mean that
two peptide sequences, when optimally aligned, such as by
30 the programs GAP or BESTFIT using default gap, share at
least 90 percent sequence identity, preferably at least
95 percent sequence identity, more preferably at least
96, 97, 98 or 99 percent sequence identity.

"Percentage amino acid identity" or "percentage amino acid sequence identity" refers to a comparison of the amino acids of two polypeptides which, when optimally aligned, have approximately the designated percentage of

5 the same amino acids. For example, "95% amino acid identity" refers to a comparison of the amino acids of two polypeptides which when optimally aligned have 95% amino acid identity. Preferably, residue positions which are not identical differ by conservative amino acid

10 substitutions. For example, the substitution of amino acids having similar chemical properties such as charge or polarity are not likely to affect the properties of a protein. Examples include glutamine for asparagine or glutamic acid for aspartic acid.

15 As further applied to polypeptides, the terms "substantial homology" or "substantial sequence homology" mean that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap, share at least 90 percent sequence homology, preferably

20 at least 95 percent sequence homology, more preferably at least 96, 97, 98 or 99 percent sequence homology.

"Percentage amino acid homology" or "percentage amino acid sequence homology" refers to a comparison of the amino acids of two polypeptides which, when optimally

25 aligned, have approximately the designated percentage of the same amino acids or conservatively substituted amino acids. For example, "95% amino acid homology" refers to a comparison of the amino acids of two polypeptides which when optimally aligned have 95% amino acid homology. As

30 used herein, homology refers to identical amino acids or residue positions which are not identical but differ only by conservative amino acid substitutions. For example, the substitution of amino acids having similar chemical properties such as charge or polarity are not likely to

affect the properties of a protein. Examples include glutamine for asparagine or glutamic acid for aspartic acid.

The phrase "substantially purified" or "isolated"
5 when referring to a protein (or peptide), means a chemical composition which is essentially free of other cellular components. It is preferably in a homogeneous state although it can be in either a dry or aqueous solution. Purity and homogeneity are typically
10 determined using analytical chemistry techniques such as polyacrylamide gel electrophoresis or high performance liquid chromatography. A protein (or peptide) which is the predominant species present in a preparation is substantially purified. Generally, a substantially
15 purified or isolated protein (or peptide) will comprise more than 80% of all macromolecular species present in the preparation. Preferably, the protein (or peptide) is purified to represent greater than 90% of all macromolecular species present. More preferably the
20 protein (or peptide) is purified to greater than 95%, and most preferably the protein (or peptide) is purified to essential homogeneity, wherein other macromolecular species are not detected by conventional techniques. As used herein, a "substantially purified" or "isolated"
25 protein (or peptide) can be synthetically or chemically produced, or recombinantly produced. A "substantially purified" or "isolated" protein or peptide as used herein is not intended to include a protein or peptide separated from an organism.

30   "Biological sample" or "sample" as used herein refers to any sample obtained from a living organism or from an organism that has died. Examples of biological samples include body fluids and tissue specimens.

High stringent hybridization conditions are selected
at about 5°C lower than the thermal melting point (Tm)
for the specific sequence at a defined ionic strength and
pH. The Tm is the temperature (under defined ionic
5 strength and pH) at which 50% of the target sequence
hybridizes to a perfectly matched probe. Typically,
stringent conditions will be those in which the salt
concentration is at least about 0.02 molar at pH 7 and
the temperature is at least about 60°C. As other factors
10 may significantly affect the stringency of hybridization,
including, among others, base composition and size of the
complementary strands, the presence of organic solvents,
i.e. salt or formamide concentration, and the extent of
base mismatching, the combination of parameters is more
15 important than the absolute measure of any one. High
stringency may be attained, for example, by overnight
hybridization at about 68°C in a 6X SSC solution, washing
at room temperature with 6X SSC solution, followed by
washing at about 68°C in a 6X SSC solution then in a 0.6X
20 SSX solution.

Hybridization with moderate stringency may be
attained, for example, by: 1) filter pre-hybridizing and
hybridizing with a solution of 3X sodium chloride, sodium
citrate (SSC), 50% formamide, 0.1M Tris buffer at pH 7.5,
25 5X Denhardt's solution; 2) pre-hybridization at 37°C for
4 hours; 3) hybridization at 37°C with amount of labeled
probe equal to 3,000,000 cpm total for 16 hours; 4) wash
in 2X SSC and 0.1% SDS solution; 5) wash 4X for 1 minute
each at room temperature and 4X at 60°C for 30 minutes
30 each; and 6) dry and expose to film.

The phrase "selectively hybridizing to" refers to a
nucleic acid molecule that hybridizes, duplexes or binds
only to a particular target DNA or RNA sequence when the
target sequences are present in a preparation of total

cellular DNA or RNA. By selectively hybridizing it is
meant that a nucleic acid molecule binds to a given
target in a manner that is detectable in a different
manner from non-target sequence under moderate, or more
5 preferably under high, stringency conditions of
hybridization. "Complementary" or "target" nucleic acid
sequences refer to those nucleic acid sequences which
selectively hybridize to a nucleic acid molecule. Proper
annealing conditions depend, for example, upon a nucleic
10 acid molecule's length, base composition, and the number
of mismatches and their position on the molecule, and
must often be determined empirically. For discussions of
nucleic acid molecule (probe) design and annealing
conditions, see, for example, Sambrook et al. 1989.
15   It will be readily understood by those skilled in
the art and it is intended here, that when reference is
made to particular sequence listings, such reference
includes sequences which substantially correspond to its
complementary sequence and those described including
20 allowances for minor sequencing errors, single base
changes, deletions, substitutions and the like, such that
any such sequence variation corresponds to the nucleic
acid sequence of the peptide/protein to which the
relevant sequence listing relates.
25   The DNA molecules of the subject invention also
include DNA molecules coding for protein analogs,
fragments or derivatives of the protein which differ from
naturally-occurring forms (the naturally-occurring
protein) in terms of the identity or location of one or
30 more amino acid residues (deletion analogs containing
less than all of the residues specified for the protein,
substitution analogs wherein one or more residues
specified are replaced by other residues, and addition
analogs wherein one or more amino acid residues are added

to a terminal or medial portion of the protein) and which
share the function of the naturally-occurring form.
These molecules include: the incorporation of codons
"preferred" for expression by selected non-mammalian
5 hosts; the provision of sites for cleavage by restriction
endonuclease enzymes; and the provision of additional
initial, terminal or intermediate DNA sequences that
facilitate construction of readily expressed vectors.

As used herein, a "peptide" refers to an amino acid
10 sequence of three to one hundred amino acids, and
therefore an isolated peptide that comprises an amino
acid sequence is not intended to cover amino acid
sequences of greater than 100 amino acids. Preferably,
the peptides that can be identified and used in
15 accordance with the subject invention (whether they be
mimotope or anti-mimotope peptides) are less than 50
amino acids in length, and more preferably the peptides
are five to 20 amino acids in length or 20-40 amino acids
in length.

20 The peptides can contain any naturally-occurring or
non-naturally-occurring amino acids, including the D-form
of the amino acids, amino acid derivatives and amino acid
mimics, so long as the desired function and activity of
the peptide is maintained. The choice of including an
25 (L)- or a (D)-amino acid in the peptides depends, in
part, on the desired characteristics of the peptide. For
example, the incorporation of one or more (D)-amino acids
can confer increased stability on the peptide and can
allow a peptide to remain active in the body for an
30 extended period of time. The incorporation of one or
more (D)-amino acids can also increase or decrease the
pharmacological activity of the peptide.

The peptides may also be cyclized, since cyclization may provide the peptides with superior properties over their linear counterparts.

As used herein, the terms "amino acid mimic" and
5   "mimetic" mean an amino acid analog or non-amino acid moiety that has the same or similar functional characteristic of a given amino acid. For instance, an amino acid mimic of a hydrophobic amino acid is one which is non-polar and retains hydrophobicity, generally by way
10   of containing an aliphatic chemical group. By way of further example, an arginine mimic can be an analog of arginine which contains a side chain having a positive charge at physiological pH, as is characteristic of the guanidinium side chain reactive group of arginine.
15   In addition, modifications to the peptide backbone and peptide bonds thereof are also encompassed within the scope of amino acid mimic or mimetic. Such modifications can be made to the amino acid, derivative thereof, non-amino acid moiety or the peptide either before or
20   after the amino acid, derivative thereof or non-amino acid moiety is incorporated into the peptide. What is critical is that such modifications mimic the peptide backbone and bonds which make up the same and have substantially the same spacial arrangement and distance
25   as is typical for traditional peptide bonds and backbones. An example of one such modification is the reduction of the carbonyl(s) of the amide peptide backbone to an amine. A number of reagents are available and well known for the reduction of amides to amines such
30   as those disclosed in Wann et al., JOC, 46:257 (1981) and Raucher et al., Tetrahedron. Lett., 21:14061 (1980). An amino acid mimic is, therefor, an organic molecule that retains the similar amino acid pharmacophore groups as is present in the corresponding amino acid and which

exhibits substantially the same spatial arrangement between functional groups.

The substitution of amino acids by non-naturally occurring amino acids and amino acid mimics as described
5 above can enhance the overall activity or properties of an individual peptide based on the modifications to the backbone or side chain functionalities. For example, these types of alterations to the amino acid substituents and peptides can enhance the peptide's stability to
10 enzymatic breakdown and increase biological activity. Modifications to the peptide backbone similarly can add stability and enhance activity.

One skilled in the art, using the above sequences or formulae, can easily synthesize the peptides. Standard
15 procedures for preparing synthetic peptides are well known in the art. The novel peptides can be synthesized using: the solid phase peptide synthesis (SPPS) method of Merrifield (J. Am. Chem. Soc., 85:2149 (1964)) or modifications of SPPS; or, the peptides can be
20 synthesized using standard solution methods well known in the art (see, for example, Bodanzsky, M., Principles of Peptide Synthesis, 2nd revised ed., Springer-Verlag (1988 and 1993)). Alternatively, simultaneous multiple peptide synthesis (SMPS) techniques well known in the art can be
25 used. Peptides prepared by the method of Merrifield can be synthesized using an automated peptide synthesizer such as the Applied Biosystems 431A-01 Peptide Synthesizer (Mountain View, Calif.) or using the manual peptide synthesis technique described by Houghten, Proc.
30 Natl. Acad. Sci., USA 82:5131 (1985).

With these definitions in mind, the subject invention provides an isolated nucleic acid molecule encoding a Plasmodium sp. chitinase. The nucleic acid molecule can be deoxyribonucleic acid (DNA) or

ribonucleic acid (RNA, including messenger RNA or mRNA),
genomic or recombinant, biologically isolated or
synthetic.

　　　The DNA molecule can be a cDNA molecule, which is a
5　DNA copy of a messenger RNA (mRNA) encoding the channel.

　　　An example of such a Plasmodium sp. chitinase is the
·Plasmodium falciparum chitinase encoded by the nucleotide
sequence as shown in SEQ ID NO:1 (this is the open
reading frame).　The amino acid sequence encoded by this
10　nucleotide sequence is shown in SEQ ID NO:3.　Another
example of such a Plasmodium sp. chitinase is the
Plasmodium gallinaceum chitinase encoded by the
nucleotide sequence as shown in SEQ ID NO:2 (this is the
open reading frame).　The amino acid sequence encoded by
15　this nucleotide sequence is shown in SEQ ID NO:4.　The
nucleotide sequence of the full gene for the Plasmodium
gallinaceum chitinase is shown in SEQ ID NO:5.

　　　The invention also provides an oligonucleotide that
is complementary to at least a portion of the mRNA
20　encoding the Plasmodium sp. chitinase.　Oligonucleotides
can be RNA or single-stranded DNA, and can be
complementary to the entire mRNA molecule encoding the
protein (i.e. of the same nucleotide length as the entire
molecule).　It may be desirable, however, to work with a
25　shorter molecule.　In this instance, the oligonucleotide
can be complementary to a portion of the entire mRNA
molecule encoding the protein.　These shorter
oligonucleotides are capable of hybridizing to the mRNA
encoding the entire molecule, and preferably consist of
30　about twenty to about one hundred nucleotides.　These
oligonucleotides can be used to reduce levels of
Plasmodium sp. chitinase, by introducing into cells an
RNA or single-stranded DNA molecule that is complementary
to at least a portion of the mRNA of the protein (i.e. by

introducing the oligonucleotide). The oligonucleotide
can base-pair with the mRNA of the protein, preventing
translation of the mRNA into protein. Thus, an
oligonucleotide can prevent translation of mRNA encoding
5 the protein into a functional protein. It may be
desirable to place the oligonucleotide downstream and
under the control of a particular promoter, so that the
oligonucleotide will prevent translation of mRNA encoding
the protein only in cells in which the particular
10 promoter functions.

More particularly, an oligonucleotide complementary
to at least a portion of mRNA encoding a Plasmodium sp.
chitinase can be used to decrease expression of a
functional channel. A cell with a first level of
15 expression of a functional Plasmodium sp. chitinase is
selected, and then the oligonucleotide is introduced into
the cell. The oligonucleotide blocks expression of
functional Plasmodium sp. chitinase, resulting in a
second level of expression of a functional Plasmodium sp.
20 chitinase in the cell. The second level is less than the
initial first level.

Oligonucleotides can be introduced into cells by any
suitable means. In one embodiment, the oligonucleotide
RNA molecule is injected directly into the cellular
25 cytoplasm, where the RNA interferes with translation. A
vector may also be used for introduction of the
oligonucleotide into a cell. Such vectors include
various plasmid and viral vectors. For a general
discussion of oligonucleotides such as antisense
30 molecules and their use, see Han et al. 1991 and Rossi
1995.

The nucleic acid molecules of the subject invention
can be expressed in suitable host cells using
conventional techniques. Any suitable host and/or vector

system can be used to express the Plasmodium sp. chitinase.

Techniques for introducing the nucleic acid molecules into the host cells may involve the use of expression vectors which comprise the nucleic acid molecules. These expression vectors (such as plasmids and viruses; viruses including bacteriophage) can then be used to introduce the nucleic acid molecules into suitable host cells. For example, DNA encoding the Plasmodium sp. chitinase can be injected into the nucleus of a host cell or transformed into the host cell using a suitable vector, or mRNA encoding the Plasmodium sp. chitinase can be injected directly into the host cell, in order to obtain expression of Plasmodium sp. chitinase in the host cell.

Various methods are known in the art for introducing nucleic acid molecules into host cells. One method is microinjection, in which DNA is injected directly into the nucleus of cells through fine glass needles (or RNA is injected directly into the cytoplasm of cells). Alternatively, DNA can be incubated with an inert carbohydrate polymer (dextran) to which a positively charged chemical group (DEAE, for diethylaminoethyl) has been coupled. The DNA sticks to the DEAE-dextran via its negatively charged phosphate groups. These large DNA-containing particles stick in turn to the surfaces of cells, which are thought to take them in by a process known as endocytosis. Some of the DNA evades destruction in the cytoplasm of the cell and escapes to the nucleus, where it can be transcribed into RNA like any other gene in the cell. In another method, cells efficiently take in DNA in the form of a precipitate with calcium phosphate. In electroporation, cells are placed in a solution containing DNA and subjected to a brief

electrical pulse that causes holes to open transiently in their membranes. DNA enters through the holes directly into the cytoplasm, bypassing the endocytotic vesicles through which they pass in the DEAE-dextran and calcium

5 phosphate procedures. DNA can also be incorporated into artificial lipid vesicles, liposomes, which fuse with the cell membrane, delivering their contents directly into the cytoplasm. In an even more direct approach, DNA is absorbed to the surface of tungsten microprojectiles and

10 fired into cells with a device resembling a shotgun.

Several of these methods, microinjection, electroporation, and liposome fusion, have been adapted to introduce proteins into cells. For review, see Mannino and Gould-Fogerite 1988, Shigekawa and Dower

15 1988, Capecchi 1980, and Klein et al. 1987.

Further methods for introducing nucleic acid molecules into cells involve the use of viral vectors. One such virus widely used for protein production is an insect virus, baculovirus. For a review of baculovirus

20 vectors, see Miller (1989). Various viral vectors have also been used to transform mammalian cells, such as bacteriophage, vaccinia virus, adenovirus, and retrovirus.

As indicated, some of these methods of transforming

25 a cell require the use of an intermediate plasmid vector. U.S. Patent No. 4,237,224 to Cohen and Boyer describes the production of expression systems in the form of recombinant plasmids using restriction enzyme cleavage and ligation with DNA ligase. These recombinant plasmids

30 are then introduced by means of transformation and replicated in unicellular cultures including procaryotic organisms and eucaryotic cells grown in tissue culture. The DNA sequences are cloned into the plasmid vector

using standard cloning procedures known in the art, as
described by Sambrook et al. (1989).

 Host cells into which the nucleic acid encoding the
Plasmodium sp. chitinase has been introduced can be used
5 to produce the Plasmodium sp. chitinase.

 Having identified the nucleic acid molecules
encoding Plasmodium sp. chitinases and methods for
expressing the Plasmodium sp. chitinases encoded thereby,
the invention further provides methods of screening a
10 substance (for example, a compound or inhibitor) for the
ability of the substance to modify Plasmodium sp.
chitinase function. In one embodiment, the method
comprises introducing a nucleic acid molecule encoding
the Plasmodium sp. chitinase into a host cell, and
15 expressing the Plasmodium sp. chitinase encoded by the
molecule in the host cell. The cell is then exposed to a
substance and evaluated to determine if the substance
modifies the function of the Plasmodium sp. chitinase.
In another embodiment, an isolated Plasmodium sp.
20 chitinase (see below) is exposed to the substance for
evaluation of whether the substance modifies the function
of the Plasmodium sp. chitinase. From these evaluations,
substances effective in altering the function of the
Plasmodium sp. chitinase can be found. Such agents may
25 be agonists or antagonists, with antagonists being
preferred herein.

 The evaluation of a cell to determine if the
substance modifies the function of the Plasmodium sp.
chitinase can be by any means known in the art. The
30 evaluation can comprise the direct monitoring of
expression of Plasmodium sp. chitinase in the host cell,
or the evaluation can be indirect.

 The nucleic acid molecules of the subject invention
can be used either as probes or for the design of primers

to obtain DNA encoding other Plasmodium sp. chitinases by either cloning and colony/plaque hybridization or amplification using the polymerase chain reaction (PCR).

Specific probes derived from SEQ ID NO:1 or SEQ ID
5   NO:2 can be employed to identify colonies or plaques containing cloned DNA encoding a member of the Plasmodium sp. chitinase family using known methods (see Sambrook et al. 1989). One skilled in the art will recognize that by employing such probes under high stringency conditions
10 (for example, hybridization at 42°C with 5X SSPC and 50% formamide, washing at 50-65°C with 0.5X SSPC), sequences having regions which are greater than 90% homologous or identical to the probe can be obtained. Sequences with lower percent homology or identity to the probe, which
15 also encode Plasmodium sp. chitinases, can be obtained by lowering the stringency of hybridization and washing (e.g., by reducing the hybridization and wash temperatures or reducing the amount of formamide employed).
20   More particularly, in one embodiment, the method comprises selection of a DNA molecule encoding a Plasmodium sp. chitinase, or a fragment thereof, the DNA molecule having a nucleotide sequence as shown in SEQ ID NO:1 or SEQ ID NO:2, and designing an oligonucleotide
25 probe for Plasmodium sp. chitinase based on the nucleotide sequence of the selected DNA molecule. A genomic or cDNA library of an organism is then probed with the oligonucleotide probe, and clones are obtained from the library that are recognized by the
30 oligonucleotide probe so as to obtain DNA encoding another Plasmodium sp. chitinase.

Specific primers derived from SEQ ID NO:1 or SEQ ID NO:2 can be used in PCR to amplify a DNA sequence encoding a member of the Plasmodium sp. chitinase family

using known methods (see Innis et al. 1990). One skilled
in the art will recognize that by employing such primers
under high stringency conditions (for example, annealing
at 50-60°C, depending on the length and specific

5 nucleotide content of the primers employed), sequences
having regions greater than 75% homologous or identical
to the primers will be amplified.

More particularly, in a further embodiment the
method comprises selection of a DNA molecule encoding

10 Plasmodium sp. chitinase, or a fragment thereof, the DNA
molecule having a nucleotide sequence as shown in SEQ ID
NO:1 or SEQ ID NO:2, designing degenerate oligonucleotide
primers based on the nucleotide sequence of the selected
DNA molecule, and employing such primers in the

15 polymerase chain reaction using as a template a DNA
sample to be screened for the presence of Plasmodium sp.
chitinase-encoding sequences. The resulting PCR products
can be isolated and sequenced to identify DNA fragments
that encode polypeptide sequences corresponding to the

20 targeted region of Plasmodium sp. chitinase.

Various modifications of the nucleic acid and amino
acid sequences disclosed herein are covered by the
subject invention. These varied sequences still encode a
functional Plasmodium sp. chitinase. The invention thus

25 further provides an isolated nucleic acid molecule
encoding a Plasmodium sp. chitinase, the nucleic acid
molecule encoding a first amino acid sequence having at
least 90% amino acid identity to a second amino acid
sequence, the second amino acid sequence as shown in SEQ

30 ID NO:3 or SEQ ID NO:4. In further embodiments, the
first amino acid sequence has at least 95%, 96%, 97%,
98%, or 99% amino acid identity to SEQ ID NO:3 or SEQ ID
NO:4.

The invention further provides an isolated DNA oligomer capable of hybridizing to the nucleic acid molecule encoding Plasmodium sp. chitinase according to the subject invention. Such oligomers can be used as

5 probes in a method of detecting the presence of Plasmodium sp. chitinase in a sample. More particularly, a sample can be contacted with the DNA oligomer and the DNA oligomer will hybridize to any Plasmodium sp. chitinase present in the sample, forming a complex

10 therewith. The complex can then be detected, thereby detecting presence of Plasmodium sp. chitinase in the sample.

The complex can be detected using methods known in the art. Preferably, the DNA oligomer is labeled with a

15 detectable marker so that detection of the marker after the DNA oligomer hybridizes to any Plasmodium sp. chitinase in the sample (wherein non-hybridized DNA oligomer has been washed away) is detection of the complex. Detection of the complex indicates the presence

20 of Plasmodium sp. chitinase in the sample. As will be readily apparent to those skilled in the art, such a method could also be used quantitatively to assess the amount of Plasmodium sp. chitinase in a sample.

For detection, the oligomers can be labeled with,

25 for example, a radioactive isotope, biotin, an element opaque to X-rays, or a paramagnetic ion. Radioactive isotopes are commonly used and are well known to those skilled in the art. Representative examples include indium-111, technetium-99m, and iodine-123. Biotin is a

30 standard label which would allow detection of the biotin labeled oligomer with avidin. Paramagnetic ions are also commonly used and include, for example, chelated metal ions of chromium (III), manganese (II), and iron (III). When using such labels, the labeled DNA oligomer can be

imaged using methods known to those skilled in the art.
Such imaging methods include, but are not limited to, X-
ray, CAT scan, PET scan, NMRI, and fluoroscopy. Other
suitable labels include enzymatic labels (horseradish
5 peroxidase, alkaline phosphatase, etc.) and fluorescent
labels (such as FITC or rhodamine, etc.).

The invention further provides an isolated
Plasmodium sp. chitinase. The protein is preferably
encoded by a nucleotide sequence as shown in SEQ ID NO:1
10 or SEQ ID NO:2 (Plasmodium falciparum and Plasmodium
gallinaceum, respectively). The protein preferably has
an amino acid sequence as shown in SEQ ID NO:3 or SEQ ID
NO:4 (Plasmodium falciparum and Plasmodium gallinaceum,
respectively). Further provided is an isolated
15 Plasmodium sp. chitinase encoded by a first amino acid
sequence having at least 90% amino acid identity to a
second amino acid sequence, the second amino acid
sequence as shown in SEQ ID NO:3 or SEQ ID NO:4. In
further embodiments, the first amino acid sequence has at
20 least 95%, 96%, 97%, 98%, or 99% amino acid identity to
SEQ ID NO:3 or SEQ ID NO:4.

It should be readily apparent to those skilled in
the art that the met residue that is present at the amino
terminal of the amino acid sequence of the Plasmodium sp.
25 chitinase (i.e., SEQ ID NO:3 and SEQ ID NO:4) and the ATG
that is present at the 5' end of the nucleotide sequence
(i.e., SEQ ID NO:1 and SEQ ID NO:2), are often added in
order to express the chitinase in a bacterial host cell.
The non-met version of the chitinase is thus specifically
30 intended to be covered by reference to SEQ ID NO:1, SEQ
ID NO:2, SEQ ID NO:3, or SEQ ID NO:4.

It should also be readily apparent to those skilled
in the art that the signal peptide is cleaved after
secretion. Both the precursoe form of the chitinase

which includes the signal peptide and the form minus the
signal peptide are specifically intended to be covered by
reference to SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, or
SEQ ID NO:4.

5   The invention further provides an antibody or
fragment thereof specific for the Plasmodium sp.
chitinase of the subject invention. Antibodies of the
subject invention include polyclonal antibodies and
monoclonal antibodies capable of binding to the
10 Plasmodium sp. chitinase, as well as fragments of these
antibodies, and humanized forms. Humanized forms of the
antibodies of the subject invention may be generated
using one of the procedures known in the art such as
chimerization. Fragments of the antibodies of the
15 present invention include, but are not limited to, the
Fab, the $F(ab')_2$, and the Fc fragments.

      The invention also provides hybridomas which are
capable of producing the above-described antibodies. A
hybridoma is an immortalized cell line which is capable
20 of secreting a specific monoclonal antibody.

      In general, techniques for preparing polyclonal and
monoclonal antibodies as well as hybridomas capable of
producing the desired antibody are well known in the art
(see Campbell 1984 and St. Groth et al. 1980). Any
25 animal (mouse, rabbit, etc.) which is known to produce
antibodies can be immunized with the antigenic Plasmodium
sp. chitinase (or an antigenic fragment thereof).
Methods for immunization are well known in the art. Such
methods include subcutaneous or intraperitoneal injection
30 of the protein. One skilled in the art will recognize
that the amount of the protein used for immunization will
vary based on the animal which is immunized, the
antigenicity of the protein, and the site of injection.

The protein which is used as an immunogen may be modified or administered in an adjuvant in order to increase the protein's antigenicity. Methods of increasing the antigenicity of a protein are well known in the art and include, but are not limited to, coupling the antigen with a heterologous protein (such as a globulin or beta-galactosidase) or through the inclusion of an adjuvant during immunization.

For monoclonal antibodies, spleen cells from the immunized animals are removed, fused with myeloma cells, such as SP2/0-Ag 15 myeloma cells, and allowed to become monoclonal antibody producing hybridoma cells.

Any one of a number of methods well known in the art can be used to identify the hybridoma cell which produces an antibody with the desired characteristics. These include screening the hybridomas with an ELISA assay, western blot analysis, or radioimmunoassay (Lutz et al. 1988).

Hybridomas secreting the desired antibodies are cloned and the class and subclass are determined using procedures known in the art (Campbell 1984).

For polyclonal antibodies, antibody containing antisera is isolated from the immunized animal and is screened for the presence of antibodies with the desired specificity using one of the above-described procedures.

In accordance with the above discussion, the subject invention further provides a method of producing an antibody specific for a Plasmodium sp. chitinase in a host. The method comprises selecting the isolated Plasmodium sp. chitinase or an antigenic portion thereof and introducing the selected Plasmodium sp. chitinase or antigenic portion thereof into a host to induce production of an antibody specific for Plasmodium sp. chitinase in the host.

The present invention further provides the above-
described antibodies in detectably labeled form.
Antibodies can be detectably labeled through the use of
radioisotopes, affinity labels (such as biotin, avidin,
5 etc.), enzymatic labels (such as horseradish peroxidase,
alkaline phosphatase, etc.), fluorescent labels (such as
FITC or rhodamine, etc.), paramagnetic atoms, etc.
Procedures for accomplishing such labeling are well known
in the art, for example see Sternberger et al. 1970,
10 Bayer et al. 1979, Engval et al. 1972, and Goding 1976.

The labeled antibodies or fragments thereof of the
present invention can be used for in vitro, in vivo, and
in situ assays to identify cells or tissues which express
Plasmodium sp. chitinase, to identify samples containing
15 Plasmodium sp. chitinase, or to detect the presence of
Plasmodium sp. chitinase in a sample. More particularly,
the antibodies or fragments thereof can thus be used to
detect the presence of Plasmodium sp. chitinase in a
sample, by contacting the sample with the antibody or
20 fragment thereof. The antibody or fragment thereof binds
to any Plasmodium sp. chitinase present in the sample,
forming a complex therewith. The complex can then be
detected, thereby detecting the presence of Plasmodium
sp. chitinase in the sample. As will be readily apparent
25 to those skilled in the art, such a method could also be
used quantitatively to assess the amount of Plasmodium
sp. chitinase in a sample. As should also be readily
apparent, such an antibody may also be used to decrease
levels of functional Plasmodium sp. chitinase, by
30 blocking the protein. Such antibodies could therefore be
used in the methods of the subject invention to prevent
infection of mosquitoes by Plasmodium sp. by interfereing
with function of Plasmodium sp. chitinase.

Further provided is a composition comprising the
Plasmodium sp. chitinase or an antigenic portion thereof
and a compatible carrier. Such compositions have
numerous uses, including the use as a vaccine. More
5 specifically, the subject invention further provides a
method of preventing transmission of malaria by a
mosquito feeding on a subject that may harbor Plasmodium
sp. organisms. The method comprises administering to the
subject an amount of a composition of the Plasmodium sp.
10 chitinase effective to induce production of an antibody
specific for Plasmodium sp. chitinase in the subject,
wherein the antibody inhibits Plasmodium sp. chitinase
and is transferred to a mosquito feeding on the subject
thereby preventing infection of the mosquito by
15 Plasmodium sp. organisms that may be harbored in the
subject.

In the methods of the invention, tissues or cells
are contacted with or exposed to the composition of the
subject invention or a compound. In the context of this
20 invention, to "contact" tissues or cells with or to
"expose" tissues or cells to a composition or compound
means to add the composition or compound, usually in a
liquid carrier, to a cell suspension or tissue sample,
either in vitro or ex vivo, or to administer the,
25 composition or compound to cells or tissues within a
subject (including the malarial parasites and mosquitoes,
and human subjects, for example).

In one embodiment, the invention provides a method
of preventing infection of mosquitoes by Plasmodium sp.,
30 the method comprising exposing the Plasmodium sp. to an
amount of a compound effective to interfere with function
of Plasmodium sp. chitinase. It should be readily
apparent that one might expose the Plasmodium sp. to the
compound directly or indirectly (for example, expose the

parasite directly, or expose the parasite via the mosquito vector or via the human host).

For example, one embodiment of the subject invention provides a method of preventing transmission of malaria

5 by a mosquito feeding on a subject that may harbor Plasmodium sp. organisms which comprises administering to the subject an amount of a compound effective to interfere with function of Plasmodium sp. chitinase in the subject, wherein the compound is transferred to a

10 mosquito feeding on the subject thereby preventing infection of the mosquito by Plasmodium sp. organisms that may be harbored in the subject. A further embodiment of the subject invention provides a method of preventing transmission of malaria by a mosquito that

15 ingests Plasmodium sp. organisms. The method comprises introducing into the mosquito an amount of a compound effective to interfere with function of Plasmodium sp. chitinase thereby preventing infection of the mosquito by ingested Plasmodium sp. organisms.

20 Interfering with (decreasing or preventing) function of Plasmodium sp. chitinase refers to modifying expression of the protein and/or modifying activity of the protein such as by inhibiting the function of the protein.

25 Function of Plasmodium sp. chitinase can be interfered with by various methods, at the gene and protein levels. In one embodiment, the function is interfered with by reducing Plasmodium sp. chitinase gene expression of the chitinase protein in the parasite.

30 This can be accomplished by exposing the parasites to a compound which reduces Plasmodium sp. chitinase gene expression. The compound could be, for example, an antisense oligonucleotide targeted to the Plasmodium sp. chitinase.

Other methods for interfering with Plasmodium sp.
chitinase gene expression could also involve site-
directed mutagenesis of the chitinase gene to prevent
expression of the chitinase, or various gene therapy
5 techniques.

Interference with function of the Plasmodium sp.
chitinase can also be accomplished by exposing the
Plasmodium sp. to a compound which inhibits function of
the Plasmodium sp. chitinase. Inhibitors could readily
10 be identified by screening methods (including the methods
described above).

Using these screening methods, several inhibitors
have been identified. These inhibitors include:
barbituric acids, 1,3,5-triazinane-2-thiones, N-
15 substituted-phenylcarbamates, and allosamizolines.

As used herein, "barbituric acids" are meant to
include derivatives and congeners of barbituric acid,
particularly derivatives and congeners of barbituric acid
that is substituted in the 5-position with an alkyl
20 group, preferably with an alkyl group which is itself
substituted with a aryl group or a heterocyclic group.
Derivatives and congeners of barbituric acid that is
substituted in the 5-position with an alkyl group are
meant to include compounds having the formula ("Formula
25 1"):
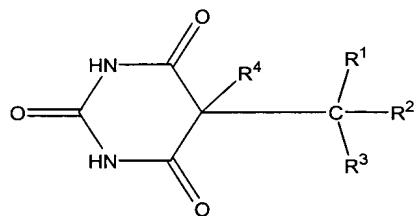
R^1, R^2, R^3, and R^4 can be independently selected from the
group consisting of a hydrogen atom, a halogen atom, an
30 alkyl group, an aryl group, and a non-aromatic
heterocyclic group. For example, R^4 can be H, one or two

of R^1, R^2, and R^3 can be hydrogen atoms, and the other two
or one of R^1, R^2, and R^3 can be selected from the group
consisting of an alkyl group, an aryl group, and a non-
aromatic heterocyclic group. Alternatively, two or more

5 of R^1, R^2, and R^3, taken together with the carbon to which
they are bonded, can form a keto (C=O) group, a
cycloalkyl group, an aryl group, or a non-aromatic
heterocyclic group. For example, R^1 can be hydrogen, and
R^2 and R^3, taken together with the carbon to which they

10 are bonded, can form a cycloalkyl group, an aryl group,
or a non-aromatic heterocyclic group, such as a cyclohex-
1-yl group, a cyclohex-2-en-1-yl group, or a cyclohex-3-
en-1-yl group; or R^1, R^2, and R^3, taken together with the
carbon to which they are bonded, can form a cycloalkyl

15 group, an aryl group, or a non-aromatic heterocyclic
group, such as a phenyl group, a naphthyl group, or a
cyclohex-1-en-1-yl group. Still alternatively, R^4 and
one or more of R^1, R^2, and R^3, taken together with the
carbons to which they are bonded, can form a cycloalkyl

20 group, an aryl group, or a non-aromatic heterocyclic
group.

"Alkyl", as used herein, is meant to include linear
alkyls (e.g., methyl, ethyl, n-propyl, n-pentyl, n-hexyl,
n-heptyl, n-octyl, n-nonyl, and the like), branched

25 alkyls (e.g., isobutyl, isopentyl, neopentyl, hex-2-yl,
hex-3-yl, hept-2-yl, hept-3-yl, and the like), and
cycloalkyls (e.g., cyclopentyl, cyclohexyl, cycloheptyl,
4-methylcyclohexyl, and the like). These alkyl groups
can be substituted or unsubstituted. When substituted,

30 suitable substituents include, for example, aryl groups,
oxo (i.e., =O) substituents, thio (i.e., =S)
substituents, halogen atoms, aldehyde groups, hydroxy
groups, alkoxy groups (examples of which include C1-C8
alkoxy groups and which are meant to include aryloxy

groups), thiol, alkylthio or arylthio groups, carboxylic
acid groups (which are meant to include carboxylic acid
derivatives, such as salts, esters, amides, etc.), amine
groups (primary, secondary, or tertiary), nitro groups,

5 sulfonic acid groups, and the like, as well as
combinations of these substituents. "Alkyl", as used
herein is also meant to include alkyl groups which
include one or more sites of unsaturation, such as a but-
3-en-1-yl group, a hex-4-en-1-yl group, a hex-4-en-2-yl

10 group, a 5-chlorohex-4-en-2-yl group, a but-3-yn-1-yl
group, a cyclohex-3-en-1-yl group, a cyclohex-2-en-1-yl
group, a cyclohex-1-en-1-yl group, and the like.
"Aryl", as used herein, is meant to include aromatic
homocyclic and heterocyclic rings and ring systems, which

15 can optionally be fused and/or contain bridged ring
systems and which can optionally be substituted with one
or more substituents, for example, selected from alkyl
groups, aryl groups, oxo (i.e., =O) substituents, thio
(i.e., =S) substituents, halogen atoms, aldehyde groups,

20 hydroxy groups, alkoxy groups (examples of which include
C1-C8 alkoxy groups and which are meant to include
aryloxy groups), thiol, alkylthio or arylthio groups,
carboxylic acid groups (which are meant to include
carboxylic acid derivatives, such as salts, esters,

25 amides, etc.), amine groups (primary, secondary, or
tertiary), nitro groups, sulfonic acid groups, and the
like, as well as combinations of these substituents.
"Non-aromatic heterocyclic group", as used herein is
meant to include all substituted or unsubstituted cyclic

30 moieties which are neither cycloalkyl groups nor aryl
groups. Examples of non-aromatic heterocyclic groups
include piperidinyl groups, morpholino groups,
hexahydropyrimidinyl groups, 2,4,6-
trioxohexahydropyrimidinyl groups (e.g., 2,4,6-

trioxohexahydropyrimidin-5-yl groups), and the like.
Bridged non-aromatic heterocyclic groups are also meant
to be included within "non-aromatic heterocyclic groups".

Illustrative barbituric acids include those having
5 the following formulae:
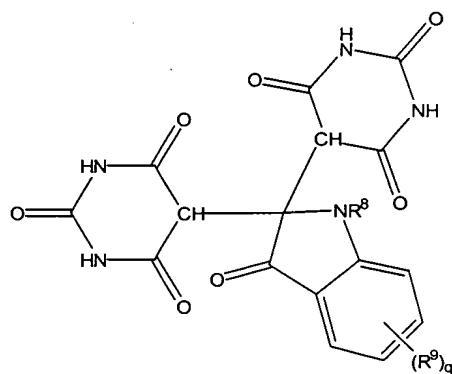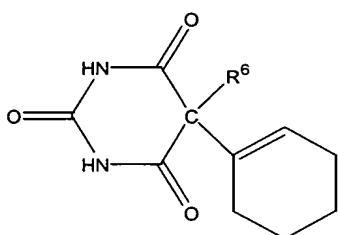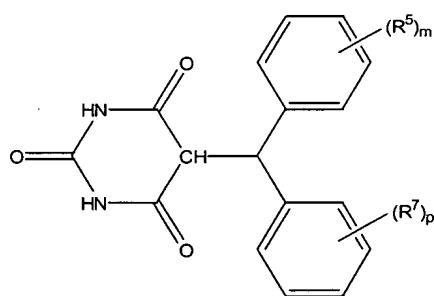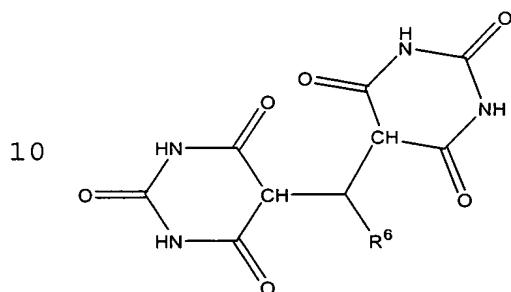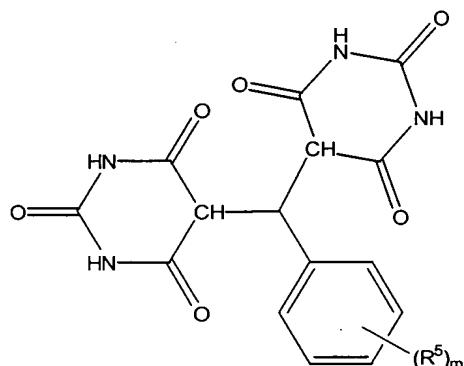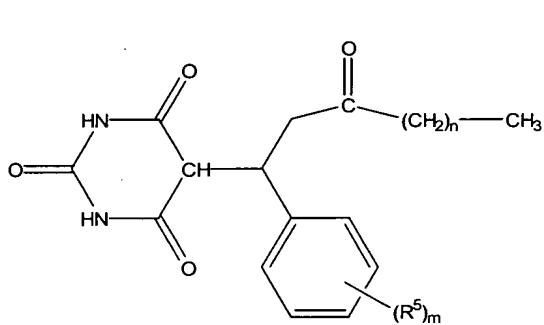
where n is an integer from 0 to 10, such as 0, 1, 2, 3,
15 4, 5, 6, 7, 9, or 10; each R^5, R^7, and R^9 is independently
selected from the group consisting of alkyl groups, aryl
groups, halogen atoms, aldehyde groups, hydroxy groups,

alkoxy groups (examples of which include C1-C8 alkoxy
groups and which are meant to include aryloxy groups),
thiol, alkylthio or arylthio groups, carboxylic acid
groups (which are meant to include carboxylic acid
5 derivatives, such as salts, esters, amides, etc.), amine
groups (primary, secondary, or tertiary), nitro groups,
sulfonic acid groups, and the like; R^6 is a hydrogen atom,
an alkyl group (e.g., a methyl, ethyl, or propyl group);
R^8 is H, alkyl, or aryl; each of m and p is independently
10 selected from 0, 1, 2, 3, 4, and 5; and q is selected
from 0, 1, 2, 3, and 4.

Barbituric acids of the types discussed above can be
obtained commercially, for example from Salor, Milwaukee,
Wisconsin or from Maybridge Chemical Company Ltd.,
15 Trevillett, Tintagel, Cornwall TL34 OHW United Kingdom.
Alternatively they can be readily prepared using
conventional methods for synthesizing barbituric acids.
Briefly, an appropriately substituted malonic acid
dialkyl ester is condensed with urea, preferably in the
20 presence of a strong base, such as sodium ethoxide. For
example, barbituric acids having Formula 1, above, can be
prepared from malonic acid dialkyl esters having the
formula ("Formula 2"):
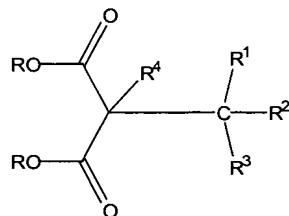
25
where R is, for example, an alkyl group, such as an ethyl
group. As a further example, barbituric acids having the
formula:
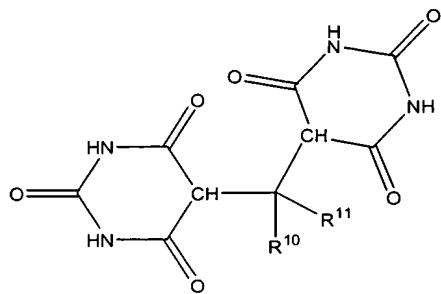
where each of R^{10} and R^{11} is, for example, a hydrogen atom, an aryl group, or an alkyl group (such as where R^{10} is hydrogen and R^{11} is an aryl or alkyl group), can be conveniently prepared in one step by reacting two equivalents of urea with a bis malonic acid dialkyl ester having the formula:
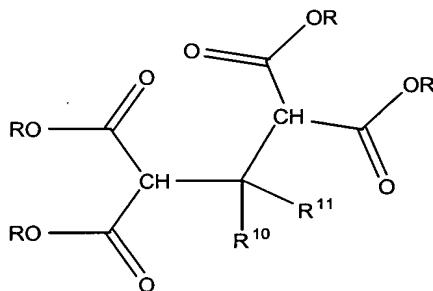
where each R represents, for example, an alkyl (e.g., ethyl) group. Further details relating to this reaction can be found, for example, in Dickey, Gray, Org. Syn., Coll. Vol. II, page 60 (1943), which is hereby incorporated by reference. Appropriately substituted malonic acid dialkyl esters and bis malonic acid dialkyl esters can be prepared by reacting malonic acid dialkyl esters using one or more appropriate electrophiles, such as an alkyl halide or an aryl isocyanate, e.g., having the formula R^4X, $XCR^1(R^2)(R^3)$, $R^{10}X$, or $R^{11}X$, where R^1, R^2, R^3, R^4, R^{10}, and R^{11} represent the substituents on the desired substituted malonic acid dialkyl esters and X represents moiety whose anion, X^-, is a good leaving group (e.g., Cl^-) or NCO.

As used herein, "1,3,5-triazinane-2-thiones" are
meant to include compounds having the formula:
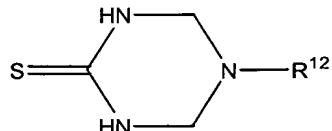
5

where R^{12} represents an alkyl moiety or a nonaromatic
heterocyclic group, as described above. Suitable
nonaromatic heterocyclic groups include, for example
azepan moieties (which may, optionally be substituted

10 with an oxo (i.e. =O) group), such as 2-oxoazepan-3-yl
moieties. Suitable alkyl moieties include unsubstituted
branched or unbranched, cyclic or non-cyclic
unsubstituted alkyl moieties (e.g., methyl, ethyl,
propyl, isopropyl, butyl, isobutyl, t-butyl,

15 1,1,3,3,tetramethylbut-1-yl, cyclopropyl, cyclopentyl,
cyclohexyl, cyclopropylmethyl, cyclopentylmethyl,
cyclohexylmethyl, 3-methylcyclohexyl, 3,3-
dimethylcyclohexyl, and 3,3,5-trimethylcyclohexyl
moieties). Other suitable alkyl moieties include aryl-

20 substituted branched or unbranched, cyclic or non-cyclic
alkyl moieties, such as imidazolylalkyl moieties (e.g.,
imidazol-1-ylmethyl, imidazol-1-ylethyl, and imidazol-1-
ylpropyl moieties); phenylalkyl moieties (e.g.,
phenylmethyl, phenylethyl, phenylpropyl, and (2,6-

25 dichlorophenyl)methyl moieties); pyridylalkyl moieties
(e.g., (2-pyridyl)methyl, (3-pyridyl)methyl, (4-
pyridyl)methyl, (2-pyridyl)ethyl, (3-pyridyl)ethyl, (4-
pyridyl)ethyl, (2-pyridyl)butyl, (3-pyridyl)butyl, and
(4-pyridyl)butyl) moieties; ((alkoxy-

30 substituted)phenyl)methyl moieties (e.g., (4-
methoxyphenyl)methyl, (3,4-dimethoxyphenyl)methyl, (4-
methoxyphenyl)ethyl, and (3,4-dimethoxyphenyl)ethyl

moieties); and thienylalkyl moieties (e.g., thien-2-ylmethyl, thien-2-ylethyl, thien-2-ylpropyl, thien-2-ylbutyl, 2-(thien-2-yl)eth-1-yl, and 2-(thien-2-yl)prop-1-yl moieties). Still other suitable alkyl moieties

5   include hydroxy-substituted and alkoxy-substituted branched or unbranched, cyclic or non-cyclic alkyl moieties, such as methoxymethyl, 4-(propoxy)but-1-yl, 4-(phenoxy)but-2-yl, 1-(hydroxy)but-2-yl, 1-(hydroxy)pent-2-yl, and 1-(hydroxy)hex-2-yl moieties. Still other

10 suitable alkyl moieties include amine-substituted branched or unbranched, cyclic or non-cyclic alkyl moieties, such as (N,N-disubstituted)aminoalkyl moieties (e.g., diethylaminomethyl, dimethylaminomethyl, diethylaminoethyl, dimethylaminomethyl, 4-(N-isopropyl-N-

15 methylamino)but-2-yl, and 2-(N-phenyl-N-methylamino)but-2-yl moieties; and (N-unsubstituted)aminoalkyl moieties (e.g., aminomethyl, aminoethyl, aminopropyl, 2-aminobut-1-yl, and 4-aminocyclohexylethyl moieties). Still other suitable alkyl moieties include branched or unbranched,

20 cyclic or non-cyclic alkyl moieties, which are substituted with nonaromatic heterocyclic groups, such as tetrahydropyrrol-2-ylmethyl, 1-(tetrahydropyrrol-2-yleth-1-yl), 3-(tetrahydropyrrol-2-ylprop-1-yl), (1-ethyltetrahydropyrrol-2-yl)methyl, tetrahydropyrrol-1-

25 ylmethyl, tetrahydropyrrol-1-ylethyl, and 2-(tetrahydropyrrol-1-yl)prop-1-yl moieties.

"1,3,5-triazinane-2-thiones" are also meant to include compounds having the formulae:

30
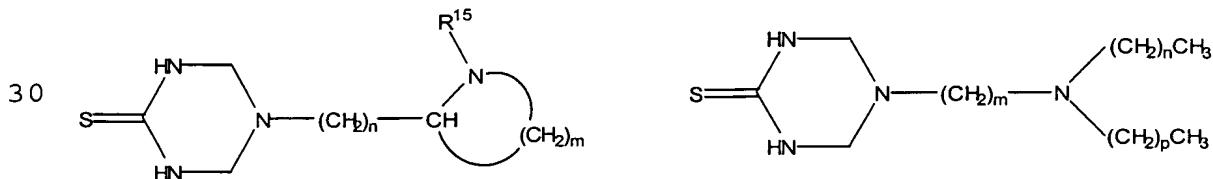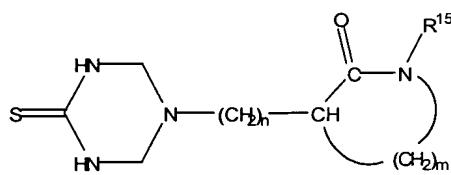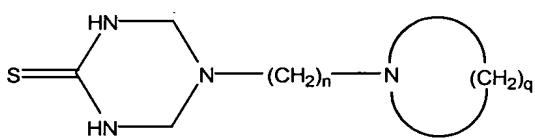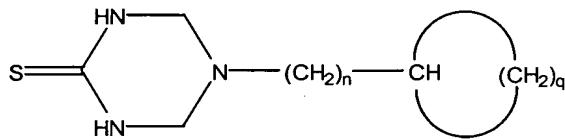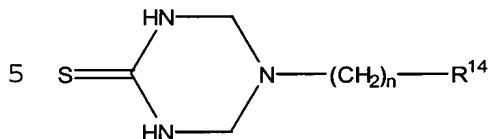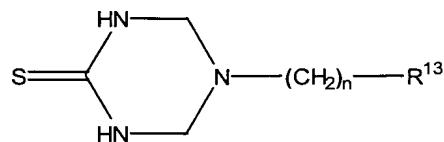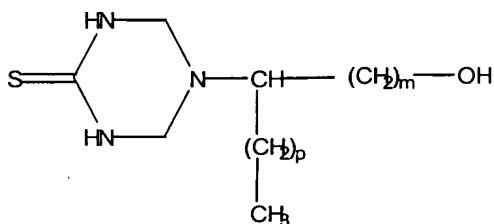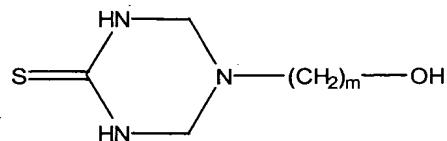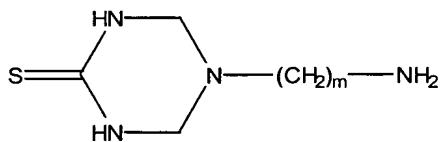
where each of n and p is independently selected from 0,
1, 2, 3, 4, 5, and 6; m is 1, 2, 3, 4, 5, or 6; q is 2,
3, 4, 5, 6, or 7; R^{13} represents a heterocyclic aromatic
group; R^{14} represents a homocyclic aromatic group; and R^{15}
represents a hydrogen atom or an alkyl or aryl group.

1,3,5-Triazinane-2-thiones of the types discussed
above can be obtained commercially, for example from
Salor, Milwaukee, Wisconsin or from Maybridge Chemical
Company Ltd., Trevillett, Tintagel, Cornwall TL34 OHW
United Kingdom.

As used herein "N-substituted-phenylcarbamates" are
meant to include compounds having the formula:
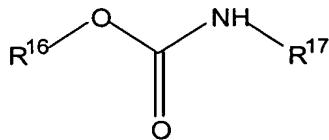
where R^{16} represents an alkyl or aryl group and R^{17} is an aryl group (as described above).

For example, the N-substituted-phenylcarbamates can have the formula:
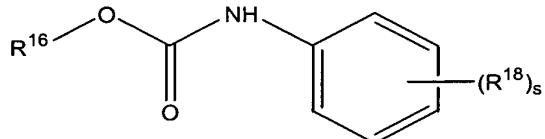
where each R^{18} independently represents alkyl groups, aryl groups, oxo (i.e., =O) substituents, thio (i.e., =S) substituents, halogen atoms, aldehyde groups, hydroxy groups, alkoxy groups (examples of which include C1-C8 alkoxy groups and which are meant to include aryloxy groups), thiol, alkylthio or arylthio groups, carboxylic acid groups (which are meant to include carboxylic acid derivatives, such as salts, esters, amides, etc.), amine groups (primary, secondary, or tertiary), nitro groups, sulfonic acid groups, and the like; and s is 0, 1, 2, 3, 4, or 5. For example, one or more of R^{18} can be a electron-withdrawing group, such as a nitro group, a halogen atom (e.g., fluoro, chloro, bromo, or iodo), a perhaloalkyl group (e.g., trifluoromethyl), or an alkoxy group (e.g., methoxy, ethoxy, etc.).

Illustratively, R^{17} can be a 3-nitrophenyl group, a 3-trifluoromethylphenyl group, a 2,5-dimethoxyphenyl group, a 2-nitro-4-chlorophenyl group, a 2-methoxy-5-nitro group, a 2-fluoro-5-nitro group, a 5-chloro-2,4-dimethoxy group, a 4-nitrophenyl group, a 4-chlorophenyl group, a 3-chlorophenyl group, and a 4-fluoro-3-nitrophenyl group.

As indicated above, R^{16} can be an aryl or alkyl group. Illustrative R^{16} aryl groups include, quinolyl

groups (e.g., 5-chloro-8-quinol-1-yl); naphthyl groups
(e.g., 5-chloro-8-naphth-1-yl); cyanophenyl groups (e.g.,
2-cyanophenyl); halogen substituted phenyl groups (e.g.,
2-bromo-4-chlorophenyl, 2-bromo-4-chloro-5-methylphenyl,
5 and 2-chloro-4-fluorophenyl); phenyl groups substituted
with carboxylic acid moieties (e.g., 2-carboxyphenyl, 2-
carbamoylphenyl, and 2-methoxycarbonylphenyl); phenyl
groups bearing unsaturated alkyl substituents (e.g., 4-
allyl-2-methoxyphenyl and 4-allylphenyl); and pyridyl
10 groups (e.g., 2-pyridyl and 5-nitro-2-pyridyl).
Illustrative R^{16} alkyl groups include, for example, alkyl
groups substituted with 2-oxo-1-imidazolidinyloxy
moieties (e.g., a 2-(2-oxo-1-imidazolidinyloxy)eth-1-yl
group) and alkyl groups substituted with a (N,N-
15 disubstituted)aminoalkyl moiety (e.g., 2-
(dimethylamino)eth-1-yl), an N-monosubstituted)aminoalkyl
moiety, or an (N-unsubstituted)aminoalkyl moiety.
Particular compounds that are illustrative of N-
substituted-phenylcarbamates include those having the
20 following formulae:

5

10
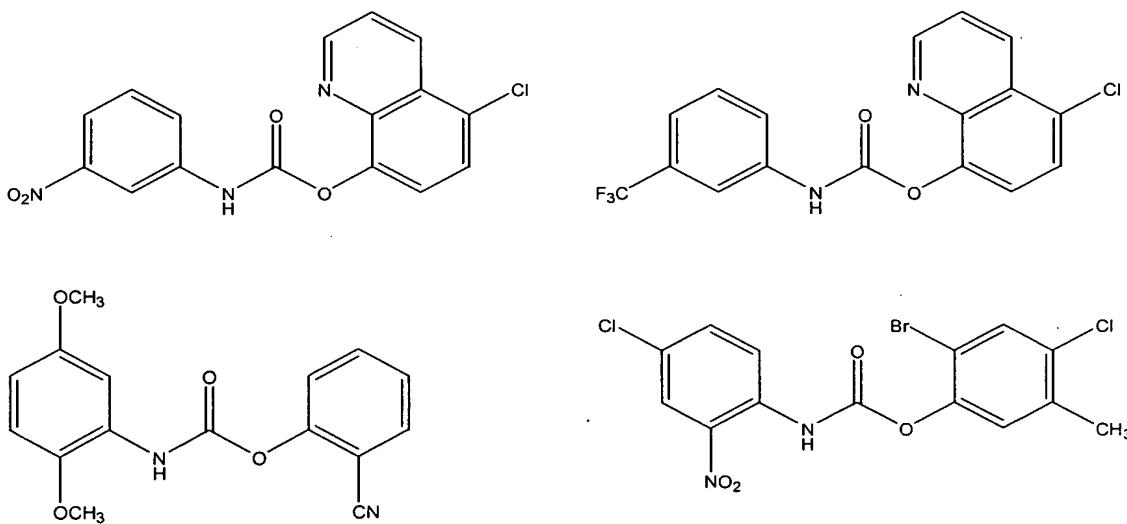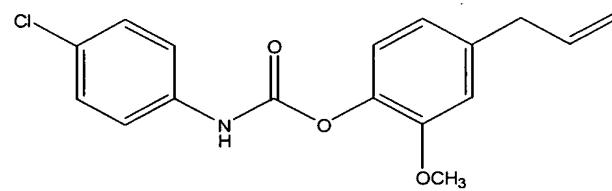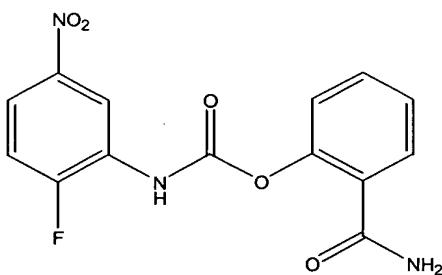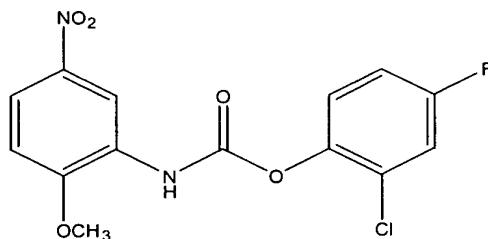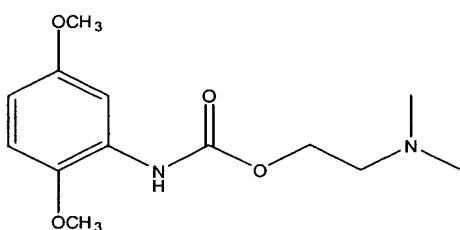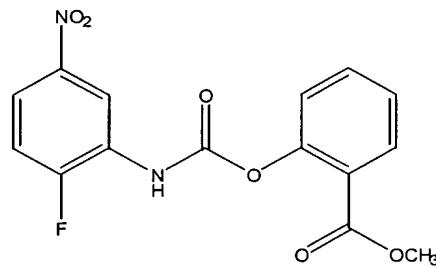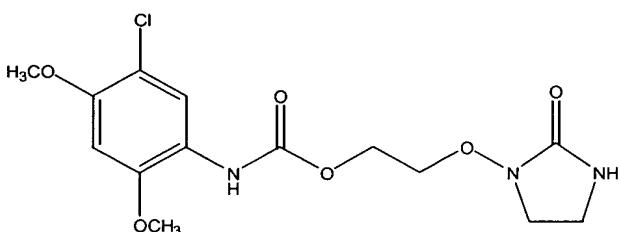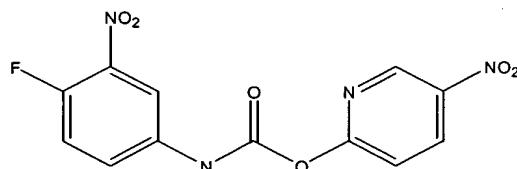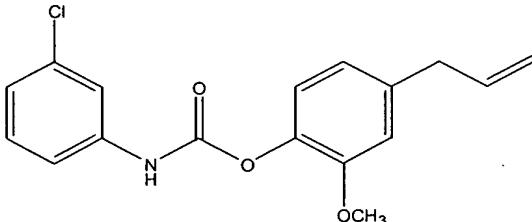
N-substituted-phenylcarbamates of the types discussed above can be obtained commercially, for example from Salor, Milwaukee, Wisconsin.

As used herein, "allosamizolines" are meant to include compounds having the following formula:
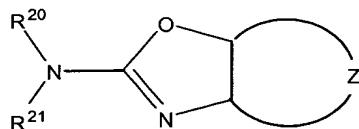
Each of R^{20} and R^{21} are independently selected from the group consisting of hydrogen, alkyl, and aryl; or R^{20} and R^{21}, together with the nitrogen to which they are bonded, represent a 3-8 membered nonaromatic heterocyclic group. Z, when taken together with the carbons to which it is bonded, represents a optionally substituted 4-10 membered cycloalkyl ring (e.g. a cyclohexane ring, a cyclopentane ring, a cyclohex-3-ene ring (fused in the 1,2 position), a 4-hydroxycyclohexane ring (fused in the 1,2 position), etc.) or an optionally substituted nonaromatic heterocyclic group (e.g., a tetrahydrofuran ring (e.g., fused in the 2,3 position), a tetrahydropyran ring (e.g., fused in the 2,3 position), a tetrahydrothiophene ring (e.g., fused in the 2,3 position), a 4-hydroxytetrahydrofuran ring (e.g., fused in the 2,3 position), a 5-hydroxyalkyl- or 5-alkoxyalkyl-tetrahydrofuran ring (e.g., fused in the 2,3 position), and a 4-hydroxy-5-hydroxymethyltetrahydrofuran ring (fused in the 2,3 position)). Specific examples of allosamizolines include those compounds having the formulae:
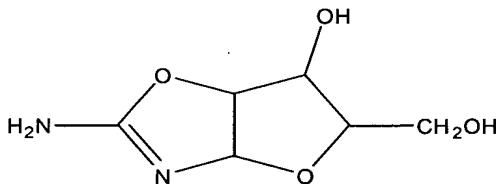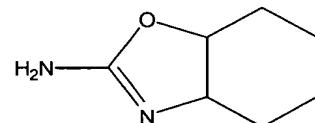
Allosamizolines of the types discussed above can be obtained commercially, for example from Salor, Milwaukee, Wisconsin or from Maybridge Chemical Company Ltd., Trevillett, Tintagel, Cornwall TL34 0HW United Kingdom.

"Barbituric acids", "1,3,5-triazinane-2-thiones", "N-substituted-phenylcarbamates", and "allosamizolines", as used herein, are meant to include their respective salts, hydrates, and the like. For example, the above-

5 described salts can be pharmaceutically acceptable salts (e.g., salts formed with hydrochloric acid, hydrobromic acid, nitric acid, sulfuric acid, phosphoric acid, citric acid, formic acid, maleic acid, acetic acid, succinic acid, tartaric acid, methanesulfonic acid,

10 p-toluenesulfonic, and the like). Other inhibitors identified using these screening methods include those having the following structural formulae:

15

20
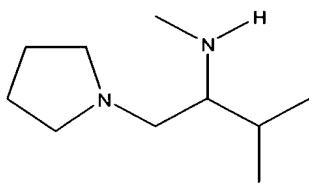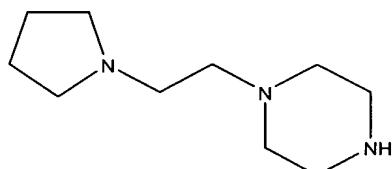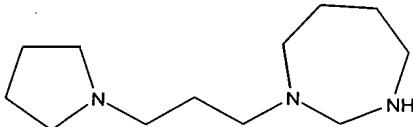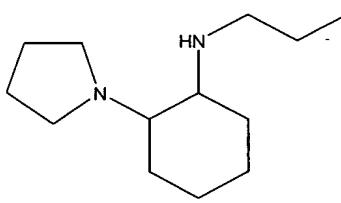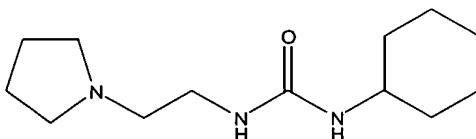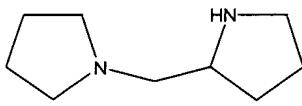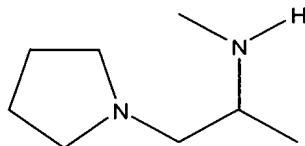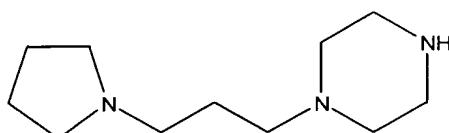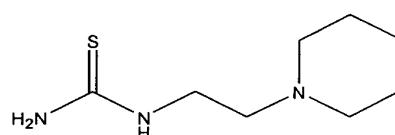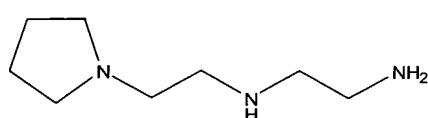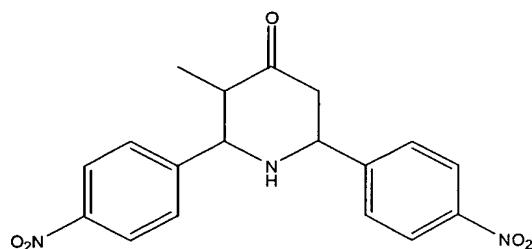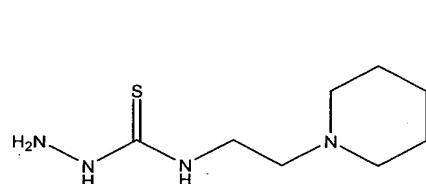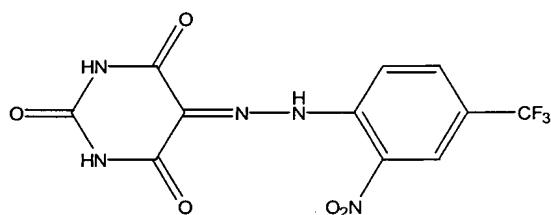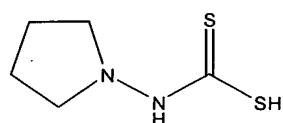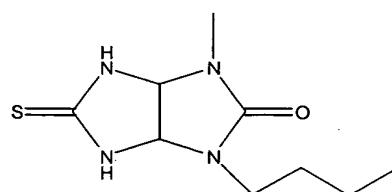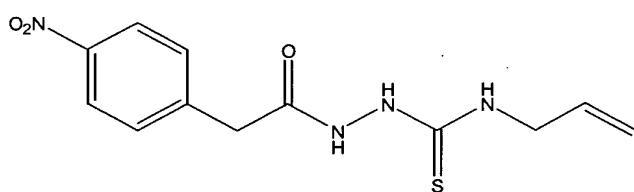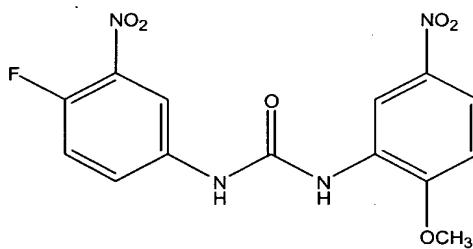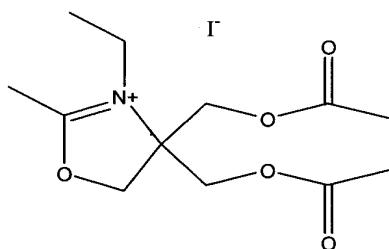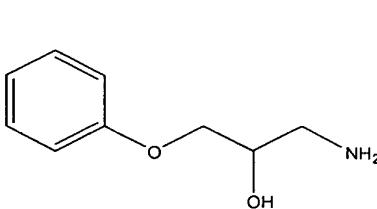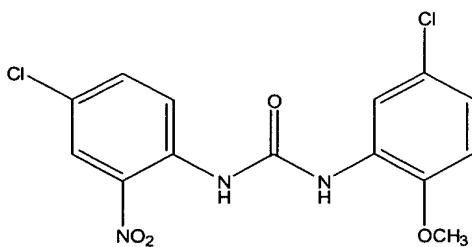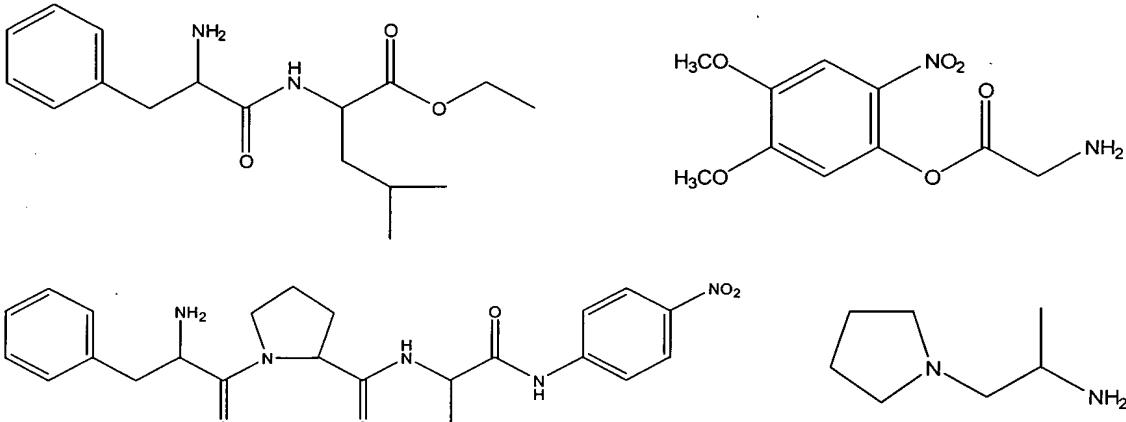
5

as well as their salts and hydrates, as discussed above.

The above described barbituric acids, 1,3,5-triazinane-2-thiones, N-substituted-phenylcarbamates,
10 allosamizolines, and other potentially useful chitinase inhibitors may exist in various isomeric, tautomeric, stereoscopic, and diasteriomeric forms, all of which are meant to be encompassed by the formulae set forth above. Moreover, each of "barbituric acids", "1,3,5-triazinane-
15 2-thiones", "N-substituted-phenylcarbamates", and "allosamizolines" is meant to include compounds that are isomerically, tautomerically, stereoscopically, and/or diasteriomerically pure as well as mixtures of such isomeric, tautomeric, stereoscopic, and diasteriomeric
20 forms.

Other potential inhibitors of chitinase can be identified by using SYBYL to construct a homology model of the PfCHT1 active site and then by using a computer docking program (such as DOCK 4.0 or those described in
25 Bugg et al., Scientific American, pages 92-98 (December 1993); West et al., TIPS, 16:67-74 (1995); and Dunbrack et al., Folding & Design, 2:27-42 (1997), which are hereby incorporated by reference) to search a database, such as the Available Chemical Database ("ACD") for

candidate chitinase inhibitors. Once potential
inhibitors of chitinase are identified, they can be
tested using conventional enzyme inhibition assays.
IC_{50}'s on the order of mM to μM (e.g., about 0.8 to about

5 2.3 mM) indicate that an inhibitor is particularly
effective. Alternatively, other potential inhibitors of
chitinase can be identified by producing crystals of
purified PfCHT1 that are suitable for x-ray
crystallographic studies, infiltrating candidate

10 chitinase inhibitor compounds into the crystal, and
analyzing the resulting complex by x-ray crystallography.
Still alternatively, candidate chitinase inhibitors can
be infiltrated into the crystal during the
crystallization process, and the resulting complexes

15 analyzed by x-ray crystallography.

The chitinase inhibitors described above can be used
to prevent malaria transmission; to prevent and treat
fungal diseases caused by, for example, Pneumocyctis
carinii, Histoplasma capsulatum, Cryptococcus neoformans,

20 Coccidioides immitis, Candida spp.; to prevent and treat
human parasitic diseases including those caused by
filaria spp., Cryptosporidium parvum, Toxoplasma gondii,
and other apicomplexans, Microsporidium spp., Leishmania
spp., Trypanosoma spp., Giardia lamblia, and Entamoeba

25 histolytica; and to prevent and treat veterinary diseases
caused by, for example, coccidan parasites, such as
Babesia spp. and Theileria spp. The chitinase inhibitors
described above can also be used to control, reduce, or
eliminate arthropod pests of agricultural and human

30 public health importance as well as veterinary arthropod
parasites, such as fleas. The inhibitors can be used,
for example, by placing them in areas that are known to
be breeding grounds or other areas of infestation of the
above described pests (e.g., kennels for fleas) or by

placing them in areas that are known habitats of animals
known to transmit (e.g., be carriers of) such pests and
diseases (stagnant water for malaria-carrying mosquitos).

In addition to chemical inhibitors, peptide
5 inhibitors could also be identified with screening
methods (for example, using phage display libraries and
other peptide screening methods).

Levels of functional Plasmodium sp. chitinase could
also be modified by use of molecules which bind to
10 transcription regulators of the chitinase gene (such as
the promoter region of the gene).

In the context of this invention "modulation" or
"modifying" generally means inhibition. This modulation
can be measured in ways which are routine in the art, for
15 example by Northern blot assay of mRNA expression or
Western blot assay of protein expression.

The compounds and/or inhibitors used in the methods
of the subject invention encompass any pharmaceutically
acceptable salts, esters, or salts of such esters, or any
20 other compound/inhibitor which, upon administration to a
subject, is capable of providing (directly or indirectly)
the biologically active metabolite or residue thereof.
Accordingly, for example, the disclosure is also drawn to
prodrugs and pharmaceutically acceptable salts of the
25 compounds and/or inhibitors used in the subject
invention, pharmaceutically acceptable salts of such
prodrugs, and other bioequivalents.

In regard to prodrugs, the compounds and/or
inhibitors for use in the invention may additionally or
30 alternatively be prepared to be delivered in a prodrug
form. The term prodrug indicates a therapeutic agent
that is prepared in an inactive form that is converted to
an active form (i.e., drug) within the body or cells

thereof by the action of endogenous enzymes or other chemicals and/or conditions.

In regard to pharmaceutically acceptable salts, the term pharmaceutically acceptable salts refers to

5   physiologically and pharmaceutically acceptable salts of the compounds and/or inhibitors used in the subject invention: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto.

10   Peptide inhibitors of chitinase can be identified by other methods also. For example, a monoclonal antibody can be prepared which specifically hybridizes to the chitinase protein, thereby interfering with activity. Once a monoclonal antibody which specifically hydridizes

15   to the chitinase protein is identified, the monoclonal (which is itself a compound or inhibitor which can be used in the subject invention) can be used to identify peptides capable of mimicking the inhibitory activity of the monoclonal antibody. One such method utilizes the

20   development of epitope libraries and biopanning of bacteriophage libraries. Briefly, attempts to define the binding sites for various monoclonal antibodies have led to the development of epitope libraries. Parmley and Smith developed a bacteriophage expression vector that

25   could display foreign epitopes on its surface (Parmley, S.F. & Smith, G.P., Gene 73:305-318 (1988)). This vector could be used to construct large collections of bacteriophage which could include virtually all possible sequences of a short (e.g. six-amino-acid) peptide. They

30   also developed biopanning, which is a method for affinity-purifying phage displaying foreign epitopes using a specific antibody (see Parmley, S.F. & Smith, G.P., Gene 73:305-318 (1988); Cwirla, S.E., et al., Proc Natl Acad Sci USA 87:6378-6382 (1990); Scott, J.K. &

Smith, G.P., Science 249:386-390 (1990); Christian, R.B., et al., J Mol Biol 227:711-718 (1992); Smith, G.P. & Scott, J.K., Methods in Enzymology 217:228-257 (1993)).

After the development of epitope libraries, Smith et

5 al. then suggested that it should be possible to use the bacteriophage expression vector and biopanning technique of Parmley and Smith to identify epitopes from all possible sequences of a given length. This led to the idea of identifying peptide ligands for antibodies by

10 biopanning epitope libraries, which could then be used in vaccine design, epitope mapping, the identification of genes, and many other applications (Parmley, S.F. & Smith, G.P., Gene 73:305-318 (1988); Scott, J.K., Trends in Biochem Sci 17:241-245 (1992)).

15   Using epitope libraries and biopanning, researchers searching for epitope sequences found instead peptide sequences which mimicked the epitope, i.e., sequences which did not identify a continuous linear native sequence or necessarily occur at all within a natural

20 protein sequence. These mimicking peptides are called mimotopes. In this manner, mimotopes of various binding sites/proteins have been found.

The sequences of these mimotopes, by definition, do not identify a continuous linear native sequence or

25 necessarily occur in any way in a naturally-occurring molecule, i.e. a naturally occurring protein. The sequences of the mimotopes merely form a peptide which functionally mimics a binding site on a naturally-occurring protein.

30   Many of these mimotopes are short peptides. The availability of short peptides which can be readily synthesized in large amounts and which can mimic naturally-occurring sequences (i.e. binding sites) offers great potential application.

Using this technique, mimotopes to a monoclonal antibody that recognizes the Plasmodium sp. chitinase can be identified. The sequences of these mimotopes represent short peptides which can then be used in

5 various ways, for example as peptide inhibitors that bind to the chitinase and decrease the activity of the chitinase. Once the sequence of the mimotope is determined, the peptide inhibitors can be chemically synthesized.

10

MATERIALS AND METHODS

A. PLASMODIUM GALLINACEUM

Preparation of P. gallinaceum Ookinete Chitinase. The 8A strain of P. gallinaceum was used to infect 4-6-week-old

15 White Leghorn chickens. A gametocyte-producing line was maintained by subpassage in chickens and periodic passage through mosquitoes. Ookinetes were cultured from purified zygotes in serum-free and protease-free M199 culture medium as described previously (Kaushal and

20 Carter 1984). Preparations routinely yielded 5-15 × 10^7 ookinetes per 5 chickens, with transformation efficiencies of 50-90%. Twenty four to 30 h cultures of ookinetes were centrifuged, and the pellet and supernatants were pooled separately and frozen at 20 °C.

25 Extracts of ookinetes were prepared by addition of 20 mM sodium phosphate, pH 6.8, to the ookinete cell pellet, usually without protease inhibitors, followed by vigorous vortexing, three cycles of freeze-thawing (dry ice to room temperature), and sonication (6 cycles for 20 s on

30 ice). For the experiment in which the time course of chitinase expression was determined with Western immunoblotting, a mixture of protease inhibitors was added directly to the fresh cell pellet in 20 mM sodium phosphate, pH 6.8 (2 mM AEBSF, 5 mM EDTA, 200 μM

N-tosyl-L-phenylalanine chloromethyl ketone, 100 μM
tosyl-L-lysine chloromethyl ketone, 1 mg/ml pepstatin, 2
mg/ml leupeptin, 2 mg/ml aprotinin, 1% Triton X-100).
Pooled supernatants were 200-fold concentrated by

5   centrifugal ultrafiltration (Centriprep 10, Amicon,
Beverly,MA), dialyzed against 20 mM sodium phosphate, pH
6.8, and frozen at 20 °C until further use.
Protein Purification and Chitinase Detection. The high
pressure liquid chromatography (HPLC) system consisted of

10   a Thermoseparation Constametric Pump and a Spectromonitor
4100 UV detector (dual wavelength at 220 and 280 nm).
The initial HPLC step consisted of 5.0 ml of pooled,
dialyzed supernatants combined with soluble ookinete
extracts injected into a quaternary ammonium anion

15   exchange column (Q column) (Vydac 300VHP575. 0.75 × 5 cm,
Hesperia, CA). Buffer A was 20 mM Tris, pH 8.0. Buffer
B was 1 M NaCl in 20 mM Tris, pH 8.0. The gradient was
developed over 30 min, from 100% Buffer A to 50% Buffer B
at a flow rate of 1.0 ml/min. Fractions were collected

20   at 1-min intervals. To individual wells of a 96-well
black microfluorimetry plate (Microfluor B, Catalog
#011-010-7205, Dynatek. Chantilly, VA), 10 μl of each
fraction was added to 160 μl of 20 mM Tris, pH 8.0, to
which 30 μl of 4-methylumbelliferyl-N,N',N"-β-D-

25   triacetylchitotrioside (4-MU GlcNAc$_3$)(Calbiochem, 125 μM
solution in water) was added. Enzyme reactions were
incubated at room temperature. A Dynatek Fluorolite 1000
(filters, excitation 365 nm and emission 450 nm) was used
for kinetic fluorescence detection for 60 min.

30   Chitinase-containing fractions (those which produced
linearly increasing fluorescence over the course of the
kinetic assay) were identified, pooled, diluted 10-fold
with saturated ammonium sulfate, pH 8.0 (to a total
volume 5 ml), and injected into a Bio-Gel TSK-phenyl 5PW

hydrophobic interaction column (Bio-Rad). Buffer A was 2
M ammonium sulfate with 20 mM Tris, pH 8.0. Buffer B was
20 mM Tris, pH 8.0. A gradient from 100% Buffer A to
100% Buffer B was developed over 30 min at a flow rate of
5 0.5 ml/min. For the anion exchange and hydrophobic
interaction steps, chitinase activity was assessed
without addition of any other buffers to the tested
fractions and without changing the pH of the final
solution before detection. Pilot experiments showed that
10 chitinase activity was readily detectable in buffers with
10 mM to 1.5 M NaCl, and at pH 5.0 to pH 8.5 in 20 mM
Tris.

Chitinase-containing fractions from the hydrophobic
interaction HPLC were pooled, mixed with an equal volume
15 of 50% acetonitrile, 0.1% trifluoroacetic acid, injected
into a reverse-phase C-18 PRP Infinity column (Hamilton,
Reno, NV), and eluted with a 10%-70% gradient of
acetonitrile, 0.1% trifluoroacetic acid at a flow rate of
0.5 ml/min.

20 **Amino-terminal Peptide Sequence Analysis.** For purified,
native, P. gallinaceum ookinete-produced chitinase, 10%
of the volume of the acetonitrile fractions from the
reverse-phase step was used to obtain amino-terminal
sequence. Recovery of the purified protein from the
25 sample tube was maximized by adding neat trifluoroacetic
acid to a final concentration of 10%. One hundred
percent of the recovered protein was applied to the
biphasic column of a Hewlett-Packard G1005A (Palo Alto,
CA), followed by a 1-ml wash with the manufacturer's
30 sample loading solution. The purified protein was then
subjected to automated Edman degradation using the
manufacturer's recommended protocols and Chemistry
Routine 3.5. For amino-terminal sequencing of
endoproteinase Lys-C-treated rPgCHT1-NT1, the cleaved

recombinant protein was run on SDS-PAGE, electroblotted
to PVDF, stained with 0.05% Coomassie Blue in 40%
methanol, 1% acetic acid, and destained with 50%
methanol. The stained band was excised and submitted to
5 automated Edman degradation on an Applied Biosystems
494/HT Procise Sequencing System in the University of
Texas Medical Branch Protein Chemistry Core Facility.
Tryptic Digestion, HPLC Separation, and Microsequencing.
To obtain amino acid sequence of internal tryptic peptide
10 fragments, the remaining 90% of the acetonitrile
fractions from the reverse-phase step was
electrophoretically purified by SDS-PAGE. 50 μl of 2$\times$
SDS-PAGE sample buffer (Novex, San Diego, CA) was added
to each 0.5-ml fraction from the reverse-phase step and
15 concentrated by vacuum centrifugation (Hetovac, Heto
Labs, Denmark). These fractions were size-fractionated
by SDS-PAGE on a 4-20% gel (Novex), which was stained
with Coomassie Blue R-250 (Bio-Rad) and destained for 4 h
with Gel-Stain Destain Solution (Novex) with three
20 changes of destaining solution. Stained bands were
excised from the gel, rinsed twice with 50% acetonitrile
in HPLC-grade water, and frozen on dry ice. Subsequent
protein sequencing steps were performed at the Harvard
University Microchemistry Laboratory (William Lane,
25 Director, Cambridge, MA). The band was subjected to
in-gel reduction, S-carboxy-amidomethylation, and tryptic
digestion (Promega) (Lane et al. 1991), and a 10% aliquot
of the resultant mixture was analyzed. Sequence
information was determined by capillary (180 μm $\times$ 15 cm
30 column, LC Packings, Amsterdam) reverse-phase
chromatography coupled to the electrospray ionization
source of a quadrupole ion trap mass spectrometer
(Finnigan LCQ, San Jose, CA). The instrument was
programmed to acquire successive sets of three scan modes

consisting of full scan MS over the m/z 395-1200 atomic
mass unit, followed by two data-dependent scans on the
most abundant ion in that full scan. These
data-dependent scans allowed the automatic acquisition of
5 a high resolution (zoom) scan to determine charge state
and exact mass and MS/MS spectra for peptide sequence
information. The remainder (90%) of the peptide mixture
was separated by microbore high performance liquid
chromatography using a Zorbax C18 1.0 × 150 mm
10 reverse-phase column on a Hewlett-Packard 1090 HPLC/1040
diode array detector. Optimum fractions were chosen
based on differential UV absorbance at 205, 277, and 292
nm, peak symmetry, and resolution and then further
screened for length and homogeneity by matrix-assisted
15 laser desorption time-of-flight mass spectrometry on a
Thermo BioAnalysis Lasermat 2000 (Hemel, UK). Strategies
for peak selection, reverse-phase separation, and Edman
microsequencing have been previously described (Lane et
al. 1991). Tryptic peptides were submitted to automated
20 Edman degradation of a Perkin-Elmer/Applied Biosystems
477A or Procise 494-HT protein sequencer (Foster City,
CA). When possible, complementary Edman degradation data
and MS/MS spectra were used to improve the final sequence
interpretation.

25 **Determination of DNA Sequence of Chitinase Gene and
Non-translated Flanking Sequence.** Degenerate
oligodeoxynucleotides were designed based on the amino
acid sequences of the following peptides (Fig. 2): GT29
(His to Lys) (SEQ ID NO:7: CA(T/C) TA(T/C) TA(T/C)
30 AA(T/C) AA(C/T) ACI GA(T/C) TA(T/C) AAA), GT33 (Asn to
Lys) (SEQ ID NO:8: AA(C/T) CCI GA(A/G) GT(A/C/T) CA(A/G)
ACI CCI AAA), and GT84 (SEQ ID NO:9: CA(C/T) AA(A/G) CCI
(C/T)TI GA(A/G) GTI GA(A/G) GA(A/G) C) (I represents
inosine). Total RNA was isolated from 24-h

post-exflagellation ookinetes with Trizol (Life
Technologies, Inc.). First-strand cDNA synthesis, using
1 μg of total RNA, was prepared using the Capfinder
system (CLONTECH, Palo Alto, CA). By using a

5 Perkin-Elmer 9600 thermal cycler and Klen-Taq DNA
polymerase (CLONTECH), the following polymerase chain
reaction (PCR) cycling protocol was used: 94 °C for 3 min
for 1 cycle; 94 °C for 30 s, 47 °C for 30 s, 68 °C for 3
min for 35 cycles; 4 °C on hold.

10   The full-length transcript was PCR-amplified using
the ookinete first-strand cDNA prepared as described
above as template (Fig. 2). To amplify the 5' end of the
transcript, an antisense nondegenerate gene-specific
oligonucleotide primer 2501 was synthesized (SEQ ID

15 NO:10: GGG TTT TCA GTT ATA GTA AGG TC) based on the
internal sequence of the PCR product generated by
degenerate oligonucleotides derived from the amino acid
sequences of GT84 and GT29; the 5' PCR primer from the
Capfinder kit (SEQ ID NO:11: AAG CAG TGG TAA CAA CGC AGA

20 GT) was used as the sense primer. Similarly, a sense
nondegenerate gene-specific primer 2503 (SEQ ID NO:12:
GAA AAA ATA TGC GAT GGG AAA GCA) was paired with the
antisense cDNA synthesis primer (T30 SEQ ID NO:13: A/C/G
A/C/G/T). The cycling protocol was as follows: 95 °C for

25 3 min for 1 cycle; then 95 °C for 30 s, 54 °C for 30 s,
68 °C for 3 min for 30 cycles; then 4 °C. PCR products
were ethanol-precipitated and resuspended in water. The
DNA was phosphorylated and the ends made blunt (10 μl of
10× React 1 buffer (Life Technologies, Inc.), 10 μl of 10

30 mM ATP, 2 μl of 10 mM dNTP (dATP, dCTP, dGTP, dTTP), 10
units of T4 DNA polymerase, 10 units of T4 kinase, water
to 100 μl, incubated at 37 °C for 60 min). The products
of this reaction were electrophoresed and then purified
from a Tris acetate/EDTA agarose gel using the Geneclean

II kit (Bio 101, San Diego, CA). The DNA was ligated
into pUC18 (SmaI-digested, bacterial alkaline
phosphatase-treated, Amersham Pharmacia Biotech),
electroporated (1.8 kV, 25 microfarads capacitance, 200

5   ohms resistance) (Gene Pulser, Bio-Rad) into
electrocompetent DH10B E. coli (Life Technologies, Inc.),
incubated in SOC (Life Technologies, Inc.) at 37 °C in a
shaking incubator for 1 h, and plated on LB/ampicillin
(100 μg/ml) plates. Plasmids from transformants were

10 isolated by alkaline lysis (Wizard MiniPrep, Promega,
Madison, WI). Clones containing the appropriately sized
insert as determined by restriction analysis were
sequenced using dye terminator reactions according to the
manufacturer's instructions (DNA Sequencing Kit, Part

15 Number 402079, Perkin-Elmer Applied Biosystems, Foster
City, CA) and analyzed by an automated ABI sequencer (ABI
Prism, 377 DNA Sequencer, Perkin-Elmer). Independent,
cloned full-length genomic DNA and cDNA PCR products were
sequenced in their entirety to verify the final sequence.

20 **Southern Blot Analysis.** P. gallinaceum gDNA (10 μg) was
digested with restriction enzymes DdeI, NcoI, EcoRI, and
HindIII (Life Technologies, Inc.) in pairs as indicated
in Fig. 3 and transferred to a nylon membrane (Hybond-N,
Amersham Pharmacia Biotech). A 42-mer

25 oligodeoxynucleotide probe (5 pmol) based on the coding
sequence of the catalytic active site (SEQ ID NO:14:
645-T AAT GAT TTT GAT TTA GAT GGT GTA GAT ATT GAC TGG GAA
CC-686) was end-labeled with [γ-^{32}P]ATP. After
prehybridizing the membrane with 6× SSC, 0.1% SDS, 50

30 μg/ml heparin, 0.1% sodium pyrophosphate (Singh and Jones
1984), the blot was hybridized for 20 h at 45 °C, washed
sequentially with 6× SSC, 0.1% SDS at room temperature
and 45 °C, respectively, and finally washed with 1× SSC,

0.1% SDS at 45 °C and exposed to Kodak X-Omat AR film
(Rochester, NY) at 70 °C for 72 h.

Expression and Preparation of Recombinant PgCHT1. The
NT1 form of rPgCHT1 PCR was amplified from a synthetic
5 DNA template constructed in Escherichia coli-preferred
codons (ECPC) (Operon, Alameda, CA). NT1 refers to the
amino-terminal sequence of the larger of the two proteins
in the purified 60-kDa chitinase chitinase doublet (Fig.
1e) that was determined by Edman degradation analysis of
10 the purified native protein. The sense primer used to
make the rPgCHT1-NT1 construct was SEQ ID NO:15: GCG CCA
TGG GTT ACG GTA GCT ATT GTG GCG; the antisense primer was
SEQ ID NO:16: GCG CTC GAG TTG CAG CGG CAG GTC CAC (the
Nco and XhoI sites, respectively, are underlined). PCR
15 cycling conditions were 94 °C x 6 min and then 18 cycles
of 94 °C x 30 s, 50 °C x 30 s and then 68 °C for 1.5 min.
The PCR product was digested and ligated into the NcoI
and XhoI restriction sites of the expression vector
pET32b. This vector expresses genes of interest as
20 fusion proteins with a 105-amino acid thioredoxin leader
sequence, amino- and carboxyl-terminal His_6 tags to
facilitate purification by nickel-chelating
chromatography, and an enterokinase cleavage site for
removal of the amino-terminal fusion partner from the
25 expressed protein. Transformants of the appropriate
construct, verified by restriction enzyme analysis and
automated sequencing of the construct in DH10B E. coli
cells (Life Technologies, Inc.), were then transfected
into E. coli strain AD494 (DE3) (Derman et al. 1993)
30 (Novagen, Madison, WI). The expression of rPgCHT1-NT1 in
AD494 (DE3) was further characterized by analyzing
protein expression by Coomassie Blue-stained SDS-PAGE
gels, Western immunoblotting, nickel-chelating
chromatography, and assays of chitinase activity to

demonstrate that protein of the appropriate size and activity was expressed.

Conditions for expressing protein for rPgCHT1-NT1 were as follows: 1) growth to A600 = 0.600 at 37 °C in a shaking incubator at 300 rpm; 2) addition of isopropyl-1-thio-β-D-galactopyranoside to 0.1 mM; 3) growth in a shaking incubator at 300 rpm at 18 °C for 16 h.

For enzymatic analysis of recombinant rPgCHT1-NT1, recombinant protein was partially purified in a single nickel-chelating chromatography step. Pellets of 2 liters of induced E. coli cells were pooled and resuspended in a lysis buffer of 20 mM Tris, 10 mM imidazole, 300 mM NaCl at 4 °C, without the addition of protease inhibitors. The cell suspension was sonicated, centrifuged at 10,000 × g, and then the supernatant was filtered through a 0.22-μm filter and batch-adsorbed to 1 ml of nickel-nitrilotriacetic acid (Ni-NTA)-agarose resin (Qiagen, Chatsworth, CA) at 4 °C. Protein was eluted from the Ni-NTA beads with 250 mM imidazole in a buffer of 20 mM Tris, pH 8.0, 300 mM NaCl, after first washing with 10 column volumes of 10 mM and then 20 mM imidazole in the same buffer.

Protease Treatment of Recombinant NT1-PgCHT1. In triplicate, aliquots of rPgCHT1 were incubated with sequence grade endoproteinase Lys-C for 1 h at 23 °C (Roche Molecular Biochemicals) or recombinant enterokinase for 4 h at 23 °C. Additional samples were heat-inactivated (10 min, 90 °C) or left untreated as controls. Amino-terminal protein sequencing was done in the University of Texas Medical Branch Protein Chemistry Core Facility using an Applied Biosystems 494/HT Procise Sequencing System.

Antibody Preparation and Immunoblotting. Two synthetic peptides were designed based on amino acid sequences found in the predicted open reading frame of the P. gallinaceum chitinase (numbered amino acids refer to Fig.

5 2): SEQ ID NO:17: 219-DLDGVDIDWEPHGK-232 (active site) and SEQ ID NO:18: 506-CDGKAAHYYNTDYKE-520 (carboxyl terminus). The peptides were chosen based on predicted antigenicity from a Kyle-Doolittle hydrophilicity plot. Peptide synthesis, carrier coupling, and structural

10 analysis of the conjugates were performed by NIAID (Twinbrook Facility, Rockville, MD). Peptide purity was verified by reverse-phase HPLC and mass spectroscopy. The peptides were coupled to keyhole limpet hemocyanin via the terminal cysteine (carboxyl-terminal peptide) or

15 lysine (active site peptide). Molar coupling ratios of peptide:carrier, as determined by amino acid analysis, were 39 for the carboxyl-terminal peptide and 413 for the active site peptide.

  Animal-use protocols for obtaining polyclonal mouse

20 antisera were approved by the Animal Care and Use Committee, NIAID, National Institutes of Health. Mice were immunized according to the following schedule: each mouse received an intraperitoneal injection of 100 μg of conjugate emulsified in 100 μl of complete Freund's

25 adjuvant (Sigma) for primary immunization, followed by 50 μg of conjugate emulsified in 100 μl of incomplete Freund's adjuvant three times at 3-week intervals.

  For immunoblotting, proteins were separated on 4-20% SDS-PAGE gels (Novex) and electroblotted to

30 nitrocellulose using the Novex Xcell BlotII module. After blocking with 5% dried skim milk in PBS, 0.05% Tween 20 (PBS-T), blots were incubated in primary polyclonal antisera at 1:1000 dilution in PBS, 0.05% Tween 20 for 1 h at room temperature. After three washes

over 30 min with PBS-T, blots were incubated in secondary
antibody (goat anti-mouse IgG heavy and light chain,
alkaline phosphatase-conjugated, Kirkegaard & Perry
Laboratories, Gaithersburg, MD) at 1:5000 dilution in
5   PBS, 0.05% Tween 20, for 1 h. After three washes over 30
min with PBS-T, the blots were developed in an alkaline
phosphatase substrate (Western Blue, Promega, Madison,
WI).

Analysis of Chitinase Activity. Chitinase activity of
10 both native and recombinant chitinases was assessed in
three ways. First, enzyme preparations were analyzed for
their ability to degrade polymeric chitin, as described
previously (Huber et al. 1991). Second, microfluorimetry
(HTS7000, Perkin-Elmer, excitation 360 nm and emission
15 465 nm) was used to measure the hydrolysis of 4-MU
GlcNAc, 4-MU GlcNAc$_2$, 4-MU GlcNAc$_3$, and 4-MU GlcNAc$_4$
(Sigma) as described previously. Initial enzyme reaction
rates were measured. Enzymatic activity is reported as
relative fluorescence units or fold change. Third, TLC
20 was used to analyze the products of recombinant or
ookinete-produced P. gallinaceum chitinase using native
chitin oligosaccharides and synthetic 4-MU derivatives of
chitin oligosaccharides as substrates. With native
chitin oligosaccharides (GlcNAc$_{1-6}$, Calbiochem), 6 μl of 5
25 mM substrate was mixed with 4 μl of 5× citrate/phosphate,
pH 3-7 (McIlvaine buffer), to which was added 10 μl of
enzyme. The reaction mixtures were incubated at 37 °C
overnight and then analyzed by TLC. 3 μl of the reaction
mixture were applied to Silica Gel-60 TLC plates, 20 × 20
30 cm (EM Science, Gibbstown, NJ) and chromatographed in
isopropyl alcohol:ethanol:water (5:2:1). The plates were
developed by spraying the plates with 10% sulfuric acid
in ethanol followed by heating at 120 °C for 10-20 min to
detect dark spots. The chromatograms were scanned on a

flat-bed scanner and images processed using Adobe
Photoshop 4.0 (Adobe Systems, Inc., San Jose, CA).
Samples of enzyme reacted with synthetic 4-MU substrates
(5 μl, containing 0.5 nmol of substrate/product) were

5 applied to 10-cm Silica Gel-60 TLC plates following
overnight incubation at 37 $^{\circ}$C. Products were separated
as above. These chromatograms were visualized with a
FLUOR-S imager using 366 nm excitation/456 nm emission
filters (Bio-Rad).

10 **Determination of pH Activity Profiles and Allosamidin**
Inhibition Curves. By using a citrate/sodium phosphate
buffer with pH ranging from 3.0 to 7.0 in 0.5 pH unit
increments, aliquots of enzyme were incubated with 4-MU
GlcNAc$_3$ (100 μM). Chitinase reaction rates were analyzed

15 by microfluorimetry. Allosamidin (Eli Lilly and Co.,
Indianapolis) was made as a 2 mg/ml stock in water and
diluted.

Computer Analysis. Analysis of DNA sequences was
performed using the Lasergene set of programs (DNASTAR,

20 Madison, WI). Homology searches were performed with
gapped BLAST, with further profile analysis performed
with PSI BLAST. The multiple alignments were generated
using the GIBBS sampling procedure. The signal sequence
was predicted using the algorithm of von Heijne (Nielsen

25 et al. 1997).

B. PLASMODIUM FALCIPARUM

Chemicals and Reagents. Routine chemicals were from
Amresco (Euclid, OH) or Sigma. Molecular biology
reagents were from Life Technologies (Gaithersburg, MD).

30 4-Methylumbelliferone (4MU) substrates were from Sigma.
Native chitin oligosaccharides (GlcNAc$_{1-6}$) were from
Calbiochem.

Identification, Cloning, and Sequencing of PfCHT1. Sense
and antisense primers derived from PfCHT1, spanning

nucleotides 154-178 and 546-570, respectively, were used
to generate a 417-bp digoxigenin (dig)-labeled probe by
PCR, which was used to screen a phage library of P.
falciparum genomic DNA (Thai isolate K1) (Goman et al.

5 1982). The probe contained the most highly conserved
regions of the gene, including the substrate-binding and
catalytic sites. Blots were developed by using an
anti-dig detection system and chemiluminescence (Roche
Molecular Biochemical, Indianapolis, IN). A HindIII

10 fragment of DNA prepared from one positive plaque was
subcloned into pUC19; this construct was called
pUC19-4.2PfCHT1. The DNA sequence of PfCHT1 was
established by automated sequencing.

Southern Blotting to Detect PfCHT1. Southern blots

15 (containing 1 μg of P. falciparum 3D7 strain DNA per
lane) were probed as described above by using the same
417-bp fragment. Final wash conditions were 2× SSC/0.5%
SDS at 45°C.

Chromosomal Localization of PfCHT1. PCR, using two

20 independent sets of PfCHT1-specific primers, was
performed on individual chromosomes isolated from
pulse-field electrophoresis-separated P. falciparum (XP5
clone; a progeny of the HB3 × Dd2 genetic cross) as
described (Su and Wellems 1999).

25 **Expression and Preparation of Recombinant P. falciparum
Chitinase.** The expression construct was prepared by
PCR-amplifying the native coding region of PfCHT1 using
pUC19-4.2PfCHT1 as template. The NcoI-containing 5'
primer was SEQ ID NO:19: GCG CCA TGG GTC ATC GAG CAC GAC

30 CAG GTG AA. The XhoI-containing 3' primer was SEQ ID
NO:20: CGCG CTC GAG ATG TAA AGA TTC TAC GAA ATA TTC. The
construct began immediately after the predicted signal
peptide cleavage site (Nielsen et al. 1997). PCR
products were restriction-digested, ligated into the NcoI

and XhoI restriction sites of the pET32b expression
vector (Novagen), and transfected into DH10B Escherichia
coli cells (Life Technologies). The correct construct
was verified by restriction digestion and automated
5 sequencing and used to transform the E. coli strain AD494
(DE3) (Novagen) for expression. Recombinant bacteria
were grown in a fermentation system (BioFlo IV; New
Brunswick Scientific), in LB with ampicillin at 100
μg/ml, as follows:(i) growth to OD_{600} = 0.800 at 37°C;
10 (ii) addition of isopropyl β-D-thiogalactoside to 0.1 mM;
and (iii) growth at 18°C for 16 hr. Pellets were treated
with 3 ml/g pellet of lysozyme (0.5 mg/ml) in 20 mM Tris,
pH 8.0/200 mM NaCl/0.1 mM PMSF and stirred at 22°C for 20
min. Triton X-100 was added to a final concentration of
15 0.1% (vol/vol), the mixture was incubated for 5 min at
22°C, and 0.1 g/ml DNase I was added with a further
10-min incubation. The cell suspension was centrifuged
at 30,000 × g for 1.5 hr, and the supernatant was
directly run over a nickel-Sepharose column (AKTA
20 Explorer System; Pharmacia). Recombinant PfCHT1
(rPfCHT1) was cleaved with recombinant enterokinase
(Novagen) and amino-terminal sequencing of the PfCHT1
product done in the University of Texas Medical Branch
Protein Chemistry Core Facility with an Applied
25 Biosystems 494/HT Procise Sequencing System.
Assessment of Chitinase Activity. rPfCHT1 activity was
assayed in glycol chitin gels by microfluorimetry and by
TLC.
Determination of Substrate Specificity, pH Profile, and
30 **Allosamidin Inhibition Curves.** Using a citrate/sodium
phosphate buffer system with pH ranging from 4.0 to 7.0
in 0.5 pH unit increments, rPfCHT1 was incubated with
native chitin oligosaccharides ($GlcNAc_{1-6}$) or 4MU-labeled
$GlcNAc_{1-4}$ substrates and analyzed by TLC or

microfluorimetry. Microfluorimetry results are reported
as initial rates of substrate hydrolysis in relative
fluorescence units.

Detection of PfCHT1 Transcripts in Mosquito Midgut

5 **Stages.** P. falciparum sexual stages were produced by
feeding infectious gametocytes (3D7 clone) to Anopheles
freeborni (Templeton et al. 1998). Mosquito midguts
containing ookinete stages were collected 24 hr postfeed.
Total RNA was extracted from a pool of 70 midguts by

10 using Trizol (Life Technologies). After treatment with
DNase I, first-strand cDNA was generated by using a
mixture of oligo(dT) and random hexamers. PCR
amplification was performed in the presence or absence of
reverse transcriptase (Superscript Preamplification

15 System; Life Technologies) to control for genomic DNA
contamination. Amplification of PfCHT1 was performed by
using the primers SEQ ID NO:21: 5' ATT ATG CTT TTA TCT
CTT GGA GG and SEQ ID NO:22: 5' AGT CTT TAC AAA ATC ACC
AAT GG. As a control, a fragment of the P. falciparum

20 gene, Pfs28, expressed by retort and ookinete stages in
the mosquito midgut (Duffy and Kaslow 1997), was
amplified by using the primers SEQ ID NO:23: 5' CAT AAC
GTT GAA TAA GGC TCG GG and SEQ ID NO:24: 5' CTA TAT GAT
GTA TCA GCC TGG TCC.

25 **Molecular Modeling of PfCHT1.** Homology models were
constructed by using sequence alignment and energy was
minimized by using SYBYL (Tripos Associates, St. Louis).
The atomic coordinates of hevamine complexed with
allosamidin (Protein Data Bank Code 1LLO) and Serratia

30 marcescens chitinase Chi A (Protein DataBank Code 1CTN)
were used as templates.

EXAMPLE I

**Purification and Microsequencing of the P. gallinaceum
60-kDa Chitinase Protein.** A mixture of supernatants and
soluble extracts of 2×10^9 P. gallinaceum ookinetes was

5 sequentially subjected to anion exchange, hydrophobic
interaction, and reverse-phase HPLC (Figs. 1a-1e). 4-MU
GlcNAc$_3$ was used as the substrate for following
endochitinase activity through the anion exchange and
hydrophobic interaction steps; acetonitrile/

10 trifluoroacetic acid in the reverse-phase step
irreversibly disrupted the chitinase activity. Coomassie
Blue staining of the major peak (fractions 62-64) in the
final purification step showed an apparently pure doublet
with apparent molecular mass of 60 kDa (Fig. 1e).

15 Fractions 62-64 contained a 60-kDa protein doublet;
fractions 65-66 contained a single 60-kDa protein and a
210-kDa protein. The 60-kDa doublet was suspected to be
a chitinase for two reasons as follows: most reported
family 18 chitinases are between 35- and 80-kDa molecular

20 mass; and polyclonal antisera raised to a synthetic
peptide derived from the active site of the Entamoeba
histolytica chitinase recognize an ookinete
stage-specific 60-kDa doublet (Vinetz and Kaslow 1998).
When an aliquot of pooled fractions 62-66 was subjected

25 to immunoblot analysis, antisera to the E. histolytica
chitinase active site recognized the 60-kDa doublet in
fractions 62-64 and the 60 and 210-kDa proteins in
fractions 65-66.

Ten percent of the pooled reverse-phase fractions
30 62-64 was analyzed by direct amino-terminal sequencing.
The remaining 90% was lyophilized in the presence of SDS,
subjected to SDS-PAGE, and stained with Coomassie Blue.
The purified doublet was excised from the gel and
subjected to microsequencing.

Direct Edman degradation of an aliquot of pooled
fractions 62-64 (Fig. 1e; 5 pmol of protein as determined
by amino acid analysis) showed two distinguishable
amino-terminal sequences (amino-terminal 1 and
5 amino-terminal 2, Fig. 3). Three peptides, GT29, GT33,
and GT84 (Fig. 3), were produced by in situ trypsin
digestion of the purified protein doublet and isolated by
reverse-phase HPLC; these protein fragments were further
analyzed by mass spectroscopy and Edman degradation.
10

EXAMPLE II

**Determination of the DNA Sequence of the P. gallinaceum
Chitinase Gene, PgCHT1, That Encodes the 60-kDa
Chitinase.** Degenerate oligodeoxynucleotide primers were
15 synthesized based on the tryptic peptide sequences GT33,
GT29, and GT84. PCR, using first-strand cDNA of mature
P. gallinaceum ookinetes as template and the pairing of
degenerate oligodeoxynucleotide primers GT33 with GT29
and GT29 with GT84, respectively, generated single
20 products that were cloned and sequenced. These PCR
fragments encoded amino acid sequences without
recognizable homology on BLAST search. Nondegenerate
gene-specific oligodeoxynucleotides, synthesized based on
the internal sequences of these PCR products, were used
25 as primers in two separate PCR reactions; these reactions
used first-strand cDNA of mature P. gallinaceum
ookinetes, prepared with the CLONTECH Capfinder system as
template (Fig. 2). The first PCR reaction used the
CLONTECH 5' Capfinder PCR primer and a gene-specific
30 antisense 3' primer; similarly, the second PCR used the
oligo(dT) primer as the 3' antisense primer and a
gene-specific 5' sense primer. When amplifying the 5'
end of the cDNA template, a single band was obtained,
cloned, and sequenced; when amplifying the 3' end,

several bands were obtained, and only the highest
molecular weight band was cloned and sequenced. These
two sequences (using PCR primers to cross the overlap
between the two gene-specific primers) were combined to
5 produce a 2508-bp cDNA corresponding to the full-length
mRNA transcript from the initiation of transcription to
the poly-adenylation tail (GenBank™ accession number
AF064079). Fig. 3 depicts the full-length translated
open reading frame. The two amino-terminal sequences of
10 the purified 60-kDa doublet determined by direct Edman
degradation and the amino acid sequences of tryptic
digest fragments GT29, GT33, and GT84 of the 60-kDa
doublet were all found in the amino acid sequence encoded
by PgCHT1.
15   PCR products derived from either gDNA or cDNA as
template had the same size and sequence, demonstrating
that the open reading frame was a single exon. A
Southern blot of P. gallinaceum gDNA probed with a 42-mer
oligodeoxynucleotide probe directed against the active
20 site was consistent with a single or low copy number copy
gene.

EXAMPLE III

Analysis of the Primary Structure of the Encoded P.
25 **gallinaceum Chitinase.** The cloned cDNA PCR product
representing a full-length chitinase cDNA contains 275 bp
of untranslated sequence at the 5' end, a 1761-bp open
reading frame encoding 587 amino acids with a predicted
molecular mass of 67,927 Da (Fig. 3), and 432 bp of
30 untranslated sequence at the 3' end. Although a Kozak
consensus sequence (Kozak 1987) is not present at the
presumptive site of the initiation of transcription, a
consensus sequence AAA(A/A)TG at the predicted
transcriptional start site typical of Plasmodium spp. is

present (Saul and Battistutta 1990). A secretory signal
sequence of 22 amino acids is predicted, followed by a
42-amino acid lysine/asparagine-rich precursor region not
found in the enzymatically active, purified 60-kDa
5 chitinase doublet. Amino-terminal sequences 1 and 2 are
colinear near the amino terminus. The two forms of the
purified chitinase are predicted to be of molecular mass
60,595 and 59,117 Da, respectively, similar to the
apparent molecular masses observed for the protein
10 doublet on SDS-PAGE (Fig. 1e).

BLAST search with the full-length P. gallinaceum
amino acid sequence revealed homologies to numerous other
chitinases; similarities were limited to the substrate
binding and catalytic active site motifs. On searching
15 the P. falciparum chromosome 14 genome data base, a
partial sequence of a P. falciparum gene encoding a
family 18 glycohydrolase catalytic domain was found.
This P. falciparum fragment was approximately 1000 bp and
encoded motifs characteristic of the substrate-binding
20 and catalytic active sites of family 18 glycohydrolases
(Henrissat and Davies 1997). This P. falciparum
chitinase has been further characterized (see Examples
which follow). The substrate-binding site (amino acids
187-194) contains the consensus sequence SEQ ID NO:25:
25 XXXSXGG, where X represents hydrophobic amino acids,
except at the amino-terminal end where there is an
unusual non-conservative isoleucine substitution for a
lysine. Both Plasmodium chitinases are typical of family
18 glycohydrolases, with one exception: uniquely the
30 Plasmodium chitinases lack a highly conserved tyrosine
(or a conservative change such as phenylalanine) at
position 229. A putative chitin-binding domain is
present that, although not sharing significant amino acid
sequence similarity with chitin-binding domains of any

eukaryotic chitinases, has significant secondary
structural homology to bacterial chitin-binding domains.

EXAMPLE IV

5 **Analysis of Different Forms of P. gallinaceum Chitinases
and Time Course of Expression.** To confirm further that
the P. gallinaceum gene sequence determined above encoded
the purified 60-kDa doublet that was identified as a
chitinase, antisera for use in Western immunoblots were

10 prepared to synthetic peptides consisting of the
catalytic active site and a peptide near the carboxyl
terminus (Fig. 3). Antibody specificity of antisera
raised against the synthetic peptides derived from the P.
gallinaceum 60-kDa chitinase was confirmed by Western

15 immunoblotting P. gallinaceum zygotes and ookinetes with
immune and preimmune sera against both synthetic
peptides; immune sera recognize proteins in ookinete but
not zygote extracts (see below), whereas preimmune sera
do not detect any proteins in the same preparations.

20 Western immunoblot of purified chitinase (fractions 65
and 66 from Fig. 1e) with antisera to the carboxyl
terminus detects only the 60-kDa doublet (Fig. 4a, right
panel). Unexpectedly, antisera to the catalytic active
site recognize, in addition to the 60-kDa doublet, a

25 210-kDa protein (Fig. 4a, left panel). This latter
result suggests that the 210-kDa protein may lack the
carboxyl-terminal epitope present in the putative
chitin-binding domain of the 60-kDa doublet. The 60-kDa
doublet and the 210-kDa protein co-eluted through a

30 multiple step purification protocol (Fig. 1e), suggesting
the possibility that P. gallinaceum ookinetes secrete at
least two structurally related forms of chitinase, a
60-kDa doublet and a 210-kDa protein.

P. gallinaceum ookinetes produce a number of
proteins (210-, 160-, 66-, 60-kDa doublet, 35-kDa) that
are stage-specifically expressed in mature ookinetes and
are not present in zygotes, as determined by analysis
5 with Western immunoblot; all of these proteins react with
antisera raised to a synthetic peptide derived from the
active site of PgCHT1 (Fig. 4b, left panel). This
antiserum lacked reactivity to P. gallinaceum zygote
extracts (Fig. 4b, left panel) and non-recombinant E.
10 coli extracts. Only a subset of these bands (160-, 66-,
and 60-kDa doublet) react with antisera directed against
the carboxyl-terminal region (Fig. 4b, right panel); the
66-kDa band (thin arrow) is the precursor to the 60-kDa
doublet as shown by Western immunoblot of ookinete
15 extracts with antisera raised to a synthetic peptide from
the region between the signal peptide and the NT1
cleavage site. The appearance of these bands correlates
with the appearance of chitinase activity during the
development of zygotes to ookinetes (Fig. 4c), consistent
20 with the hypothesis that these bands are those of
chitinases.

EXAMPLE V

P. gallinaceum Ookinetes Produce at Least Two
25 **Chromatographically and Immunologically Distinct
Chitinases.** Initially, only one broadpeak of
endochitinase activity was isolated by anion exchange
chromatography of P. gallinaceum ookinete extracts. When
a slower flow rate and more gradual salt gradient were
30 used to chromatograph ookinete extracts by strong anion
exchange HPLC, two peaks of endochitinase activity
(hereafter referred to as peak 1 and peak 2), as assessed
by hydrolysis of 4-MU GlcNAc$_3$ were reproducibly resolved
(Fig. 5a).

The separate peaks of chitinase activity were
concentrated, subjected to SDS-PAGE under both reducing
and non-reducing conditions, and Western immunoblotted
using antisera that recognize the catalytic active site
5 and carboxyl terminus (Figs. 5b-5e). Several proteins
reacted with antibody to the active site of PgCHT1.
Western immunoblot of peak 1 (fractions 35-37) with
active site antisera demonstrated the 60-kDa chitinase
doublet both under non-reducing (Fig. 5b) and reducing
10 (Fig. 5d) conditions. In addition, a 35-kDa protein in
peak 1, of unknown function, reacted with the active site
antisera. This 35-kDa protein is stage-specifically
expressed in ookinetes, is not found in zygotes (Fig. 4b,
left panel), and could be either a degradation product of
15 another chitinase protein, the product of another
chitinase gene, or simply an unrelated protein. Western
immunoblot of peak 2 (fractions 42-44) with active site
antisera demonstrated a predominant band of 210 kDa under
non-reducing conditions (Fig. 5b) that, under reducing
20 conditions (Fig. 5d), was not detected and was replaced
by a 35-kDa protein. In addition, in fraction 42, a band
of 160 kDa reacted with antiserum to the active site but
not the carboxyl terminus (Figs. 5b-5e). This 160-kDa
protein is stage-specifically expressed in ookinetes and
25 is not detectable in zygotes (Fig. 5b, both panels).
Additional resolution of this second peak of chitinase
activity with additional anion exchange chromatography
and enzymatic analysis suggests that both the 210- and
the 160-kDa proteins are chitinases. The active site
30 antisera recognized both the 210- and 35-kDa bands, but
the carboxyl-terminal antisera recognized neither (Figs.
5b and 5c). This finding suggests that the
carboxyl-terminal epitope is absent in the 210- and
35-kDa bands in the second peak of chitinase activity, as

well as the 35-kDa protein in the first peak of chitinase activity.

EXAMPLE VI

5 **Sites of Action of Endoproteinase Lys-C on the 60-kDa P. gallinaceum Chitinase.** P. gallinaceum chitinase has been reported to be secreted as an inactive zymogen that mosquito midgut proteases activate to a fully active enzyme (Shahabuddin et al. 1993); this finding has become

10 established in the literature (Shahabuddin et al. 1993; Shahabuddin 1998; Shahabuddin et al. 1996; Tellam et al. 1999). The serine protease endoproteinase Lys-C (Endo Lys-C) was reported to increase the P. gallinaceum chitinase activity in culture supernatants up to 13-fold

15 (Shahabuddin et al. 1993). To characterize further this phenomenon, Western immunoblotting of native, ookinete-secreted chitinase was used (Figs. 61-6d) and amino-terminal sequencing of Endo Lys-C-cleaved rPgCHT1 to delineate the sites where Endo Lys-C cleaves the

20 60-kDaP. gallinaceum chitinase.

Concentrated ookinete supernatants were treated with Endo Lys-C and the reactions terminated with the serine protease inhibitor, AEBSF. It was found that chitinase activity, as assessed by 4-MU GlcNAc$_3$ hydrolysis, was

25 unaffected by treatment with Endo Lys-C, in contrast to the results of others (Shahabuddin et al. 1993). Immunoblots were performed on the reaction mixtures, under non-reducing and reducing conditions, with polyclonal antisera to the active site- and carboxyl

30 terminus-derived peptides (Figs. 6a-6d). Endo Lys-C had two effects on the 60-kDa doublet as follows: 1) both bands of the doublet were cleaved, resulting in a single band running at a slightly smaller molecular mass than the bottom band of the doublet; and 2) a time-dependent

disappearance of the carboxyl-terminal epitope occurred.
Antisera to the active site demonstrated that a single
full-length chitinase remained after Endo Lys-C treatment
under non-reducing conditions. Under reducing conditions
5 a small fragment was removed, resulting in a slightly
faster migration of the processed chitinase (Figs. 6a-6d,
right top arrowhead). The precise epitope recognized by
the carboxyl-terminal antisera, which demarcates the
putative chitin-binding domain, contains a predicted Endo
10 Lys-C site at Lys^{509}-Ala^{510}, consistent with the
experimental findings. Cys^{506}, at the amino terminus of
the epitope, may form a disulfide bridge with the only
cysteine downstream from it, Cys^{557} (Fig. 3).

Since Endo Lys-C converts the 60-kDa chitinase
15 doublet to a slightly smaller single band, the
amino-terminal site of action of this protease was
determined. After treatment of rPgCHT1-NT1 with Endo
Lys-C, the cleaved product was separated by SDS-PAGE,
transferred to a PVDF membrane, and analyzed by Edman
20 degradation. This analysis showed that Endo Lys-C
cleaves rPgCHT1 on the carboxyl-terminal side of Lys^{86},
just downstream from the NT2 cleavage site (Fig. 3).
Immunoblot of Endo Lys-C-treated rPgCHT1 with the
anti-carboxyl-terminal antiserum showed loss of the
25 epitope, similar to that found when native
ookinete-produced 60-kDa chitinase was treated with Endo
Lys-C (Figs. 6a-6d).

EXAMPLE VII

30 **Expression of Recombinant PgCHT1**. Numerous constructs of
native codon-based PgCHT1, using different vectors
expressed under a variety of temperature conditions and
isopropyl-1-thio-β-D-galactopyranoside concentrations in
E. coli host cells, did not produce more than ~5 μg of

recombinant protein/liter-induced E. coli cells.
Undetectable quantities of recombinant protein were
obtained when attempting to express similar constructs in
a well characterized Saccharomyces cerevisiae expression

5 system (Kaslow and Shiloach 1994). Because the A + T
codon bias of this gene (70.6%) was suspected to be the
primary barrier to producing recombinant protein, a
synthetic PgCHT1 gene was constructed using E.
coli-preferred codons and used as a PCR template for

10 making the rPgCHT1-NT1 construct (see Materials and
Methods). Recombinant PgCHT1-NT1 (depicted schematically
in Fig. 7a), expressed with a construct synthesized in E.
coli-preferred codons and expressed in E. coli AD494
(DE3) cells, produced ~5-10 mg of recombinant

15 protein/liter of induced cells, of which ~1-3% was
soluble and active. Chitinase activity was readily
detectable in crude soluble extracts of the cells. When
the same constructs were expressed in E. coli BL21 (DE3)
cells, no chitinase activity was detected, despite a

20 comparable total quantity of protein produced. Western
immunoblot analysis of protein obtained by a Ni-NTA
purification step demonstrated that the eluted, soluble
proteins were >90% PgCHT1. Approximately 20% of the
soluble protein in eluted fractions was rPgCHT1-NT1 of

25 the predicted length; the rest were aggregated and
truncated forms of rPgCHT1.

EXAMPLE VIII

P. gallinaceum Chitinases Degrade Polymeric Chitinase and

30 **Have Identical Substrate Preferences and Reaction Product**
Profiles. A previous report demonstrated that several
bands of chitinolytic activity were present in crude
extracts and culture supernatants of P. gallinaceum
ookinetes, as determined in a glycol chitin activity gel

(Huber et al. 1991). rPgCHT1-NT1, whether or not treated with proteases, also degraded polymeric chitin in a glycol chitin activity gel (Fig. 7b); quantitation of chitin degradation was not possible from this experiment.

5 As negative controls, Endo Lys-C alone and heat-inactivated rPgCHT1NT1 had no detectable chitinase activity in the activity gel. The ability of rPgCHT-NT1 to cleave 4-MU GlcNAc$_3$ was then assessed. When rPgCHT1-NT1 was treated with enterokinase to remove the

10 thioredoxin fusion partner, there was no change in enzymatic rate of 4-MU GlcNAc$_3$ (Fig. 7c). However, to exploit the finding that Endo Lys-C cleaves PgCHT1 just downstream of the NT2 cleavage site, rPgCHT1 was treated with Endo Lys-C. This protease treatment increased

15 chitinase activity 73-fold, using the 4-MU GlcNAc$_3$ substrate (Fig. 7c). As an additional control to show that the Endo Lys-C and enterokinase did in fact cleave the recombinant protein, Western immunoblot analysis performed before and after protease treatment

20 demonstrated the appropriately sized cleavage products.

To characterize the substrate specificity and reaction product profiles of P. gallinaceum chitinases, the activities of ookinete-produced chitinase and rPgCHT1-NT1 were assessed with native chitin oligomers

25 (Fig. 8) and 4-MU GlcNAc substrates (Figs. 9a-9d). P. gallinaceum ookinete-produced chitinase (peak 1, peak 2, and unfractionated extracts) and rPgCHT1-NT1 had identical substrate preferences and reaction product profiles, with the exception that the crude extract had

30 an N-acetylglucosaminidase activity not found in peak 1 or peak 2 of anion-exchanged chromatography fractionated ookinete extracts (Figs. 8 and 9a-9d). Both parasite-produced chitinase and rPgCHT1-NT1 hydrolyzed 4-MU GlcNAc$_3$ and 4-MU GlcNAc$_4$ (Figs 9a-9d). Treatment of

rPgCHT1-NT1 with Endo Lys-C had no effect on the pattern of substrates preferred by the enzyme nor on the reaction product profile. Regardless of whether rPgCHT1-NT1 was treated with Endo Lys-C, it did not hydrolyze 4-MU GlcNAc or 4-MU GlcNAc$_2$. The kinetics of Endo Lys-C-treated rPgCHT1-NT1 on 4-MU GlcNAc$_3$ and 4-MU GlcNAc$_4$ were analyzed. By using a Lineweaver-Burk plot, the Km values of Endo Lys-C-treated rPgCHT1 were determined to be 140 μM at pH 6.0 for 4-MU GlcNAc$_3$ and 100 μM at 6.0 for 4-MU GlcNAc$_4$. The Km values found for other recombinant and native chitinases are similar. For example, native and recombinant Brugia malayi chitinase has Km = 40 μM (Venegas et al. 1996) and purified native S. marcescens ChiA and ChiB have Km = 40 μM (Brurberg et al. 1996).

EXAMPLE IX

P. gallinaceum Chitinases Have Different pH Activity Profiles and Susceptibility to the Inhibitor Allosamidin.
pH activity profiles were determined by microfluorimetry for peaks 1 and 2 of chitinase activity and for Endo Lys-C-treated rPgCHT1 (Fig. 10a) (peak 1 contains PgCHT1), using 4-MU GlcNAc$_3$ as substrate. Peak 2 chitinase activity had a broad pH optimum of pH 4.0-5.0. Peak 1 chitinase activity was optimal at pH 5.0. Similar to peak 1, rPgCHT1 also had a pH optimum of 5.0. This reproducible difference in pH activity profiles suggests that the two peaks of chitinase activity are comprised of chitinases with different amino acids present in the catalytic sites of the enzymes and thus are products of different genes.

To confirm and extend the suggestion that P. gallinaceum ookinetes may secrete at least two chitinases derived from different genes, the sensitivity of each of the two peaks of chitinase activity and rPgCHT1 to the

chitinase inhibitor allosamidin was determined (Fig. 10b). The IC_{50} value estimated for peak 1 and rPgCHT1 were similar (7 and 12 μM, respectively). In contrast, the IC_{50} value for peak 2 was 0.3 μM, about 30-fold less

5 than that found for peak 1 or rPgCHT1. The allosamidin inhibition data provides additional evidence for a second P. gallinaceum chitinase gene and that peak 1 is the product of the PgCHT1 gene. The 1 and 0.1 mM concentrations of allosamidin used in previous studies to

10 block oocyst development (Shahabuddin et al. 1993) far exceed the IC_{50} values for both peaks of chitinase activity and would completely inhibit chitinase activity in ookinete extracts.

15   **EXAMPLE X**

Plasmodium gallinaceum. The above Examples report the purification of a 60-kDa P. gallinaceum ookinete-secreted chitinase and characterize the gene, PgCHT1, encoding it. The experiments presented identify at least two

20 developmentally regulated chitinases expressed by P. gallinaceum ookinetes. Both are inhibited by allosamidin at concentrations far less than those used in in vivo studies for blocking ookinete penetration of the mosquito peritrophic membrane. Both are secreted and act as

25 endochitinases, a property that would be expected of enzymes that allow the ookinete to penetrate and traverse the PM in the mosquito midgut.

At least two chitinase activities are separable by HPLC. The first, encoded by the gene, PgCHT1, was

30 identified from peak 1 as a 60-kDa doublet (Fig. 1), which is composed of two forms of the protein, NT1 and NT2, which differ in size by 14 amino acids (Fig. 3). The NT1 form of PgCHT1, expressed as a recombinant protein in E. coli, has chitinase activity that is

increased 73-fold by treatment with Endo Lys-C. The Endo
Lys-C cleavage site is between eight and nine amino acid
residues downstream from the amino terminus of the NT2
form.

5   Peak 2 chitinase hydrolyzes 4-MU derivatives in a
pattern similar to that produced by peak 1 and rPgCHT1.
However, peak 2 chitinase has a distinct pH activity
profile and is about 30-fold more sensitive to
allosamidin (Fig. 10b) than rPgCHT1 and native peak 1
10 chitinase. Under reducing and denaturing conditions, a
number of proteins, including a 35-kDa protein in peak 2,
were identified by Western immunoblotting that increase
in expression in parallel with an increased chitinase
activity (Figs. 4a-4c and 5a-5e). The 35-kDa protein
15 reacts with antisera prepared from a peptide from the
catalytic domain of PgCHT1, but not with antisera from a
carboxyl-terminal peptide of PgCHT1. Collectively, the
different enzymatic properties and immunological
reactivity of the peak 2 chitinase support that this
20 chitinase is the product of a different gene, PgCHT2.
Under non-reducing conditions, peak 2 chitinase migrates
as a ~210-kDa protein.

An important goal of research on the malaria
parasite chitinases is to develop novel ways to interrupt
25 malaria transmission. With the cloning of a full-length
P. gallinaceum chitinase gene, recombinant chitinase can
be synthesized and tested as a transmission-blocking
vaccine in an in vivo avian malaria transmission model.

30   **EXAMPLE XI**

**Characterization of the Full-Length P. falciparum
Chitinase Gene, PfCHT1.** A partial sequence of PfCHT1 was
identified initially in the P. falciparum genome project.
A 4.2-kb fragment containing the full-length PfCHT1 gene

was obtained from screening a phage library of P.
falciparum genomic DNA. PfCHT1 has a predicted
single-exon ORF of 1,134 bp, with a 71.2% A/T content.
Sequence translation predicts a protein of 378 aa and an
5 expected molecular mass of 42,792 Da. The 15 bp 5' to
the predicted translational initiation site are SEQ ID
NO:26: (-15) AATAAATATATAAAC (-1), consistent with
sequences reported to flank the translational start sites
of yeast, P. falciparum, and other protozoa (Yamauchi
10 1991; Saul and Battistutta 1990; Hamilton et al. 1987).
A secretory signal peptide sequence of 28 aa is predicted
to be present at the amino terminus (Fig. 11) (Nielsen et
al. 1997), which further supports the assignment of the
translational initiation codon. In the 400 bp of 90%
15 AT-rich DNA 5' to the predicted start codon, no intron
splice sites, exons, or start methionines are
identifiable but stop codons are present in all three
reading frames. PfCHT1 contains substrate binding and
catalytic sites typical of family 18 chitinases.
20 Comparison of PfCHT1 with PgCHT1 (Fig. 11) suggests that
PfCHT1 lacks a chitin-binding domain. An encoded
chitin-binding domain is not detectable within 1,500 bp
of sequence downstream of the predicted stop codon within
the 4.2-kb genomic clone that contains PfCHT1. In
25 contrast to PgCHT1, PfCHT1 does not contain an amino acid
sequence consistent with a proenzyme domain or sequences
homologous to the NT1 or NT2 loops found in PgCHT1 (see
Fig. 11).
    Hybridization of a Southern blot with the same
30 417-bp digoxigenin-labeled fragment used to screen the
genomic phage library demonstrated a single band on
genomic DNA digested by five separate restriction enzymes
(Fig. 12a). This is consistent with a single- or
low-copy-number gene. Genomic DNA restricted by BglII

yielded one predominant band of 8.2 kb and two smaller, less prominent, bands of 6-6.5 kb of uncertain significance. PfCHT1 has been localized to chromosome 12 (Fig. 12b).

5   To determine whether stages of P. falciparum developing within the mosquito midgut express PfCHT1, total RNA was prepared and analyzed for the presence of PfCHT1 transcript by reverse transcription-PCR (Fig. 12c). At 24 hr postblood meal, a PfCHT1 message was

10   detectable in P. falciparum-infected mosquito midguts. Sequencing of the amplicons showed that they were identical to PfCHT1. A PfCHT1 message was not detected in midguts taken from mosquitoes 24 hr after a noninfectious blood meal. These findings demonstrate

15   that P. falciparum ookinetes within the mosquito midgut transcribe PfCHT1.

PfCHT1 transcription was examined in cDNA libraries of other P. falciparum stages. PfCHT1 and the zygote/ookinete marker Pfs25 were both detected in

20   gametocyte cDNA as well as in two asexual blood-stage cDNA libraries known to contain gametocyte transcripts. These results with Pfs25 are consistent with previous observations, which also noted that Pfs25 protein expression was delayed until after exflagellation and

25   fertilization in the mosquito midgut (Kaslow et al. 1988). Zygotes of P. gallinaceum also contain PgCHT1 mRNA but undetectable PgCHT1 protein or chitinase enzymatic activity. No PfCHT1 mRNA was detected in a sporozoite cDNA library (Fidock et al. 1998).

30

EXAMPLE XII

Expression of Enzymatically Active PfCHT1. rPfCHT1 was expressed as a thioredoxin (trx) fusion protein in the expression plasmid pET32b by using as host cells the E.

coli mutant nonreducing strain AD494, which allows for intracytoplasmic formation of disulfide bonds. The trx-rPfCHT1 fusion protein was constructed with a hexahistidine (His$_6$) tag at both amino and carboxyl

5 termini. The expressed chitinase began at the amino acid immediately after the predicted signal peptide cleavage site and included the remainder of the ORF. Chitinase activity was readily found in crude, soluble extracts of induced recombinant bacteria, as detected by hydrolysis

10 of 4MU-chitotrioside. When the same construct was expressed in E. coli BL21 cells, chitinase activity was not detected, despite a comparable total quantity of recombinant protein produced. Chitinase activity, by the measures described here, was not detectable in E. coli

15 strains AD494 or BL21 transformed with the pET32b vector with no insert. Cell lysates from a 16-liter fermentation of rPfCHT1 were chromatographed with an imidazole step gradient on a nickel-Sepharose column (Fig. 13a), yielding a trx-rPfCHT1 fusion protein of >95%

20 purity as determined by Coomassie blue staining (Fig. 13b). Western immunoblot and amino-terminal sequencing confirmed the identity of the recombinant protein. The amino-terminal His$_6$ tag was found to be responsible for binding to nickel Sepharose; the carboxyl terminal His$_6$

25 sequence was found not to bind to nickel Sepharose. Therefore, any chitinase activity eluting from the nickel Sepharose column at 250 mM imidazole must have an intact amino-terminal His$_6$ tag, indicating that no unexpected proteolytic degradation of trx-rPfCHT1 had occurred to

30 yield a shorter-length, enzymatically active rPfCHT1. The fused trx sequence was released by treatment with enterokinase, which yielded rPfCHT1 with a molecular mass of 39 kDa (Fig. 13c). The final yield was 7 mg of enterokinase-cleaved rPfCHT1 from a 16L fermentation run.

This 39-kDa protein had robust chitinase activity as
determined by hydrolysis of 4MU-GlcNAc$_3$. Amino-terminal
sequencing of this 39-kDa band gave the amino acid
sequence SEQ ID NO:6: ARPGE, the amino terminus of
5 rPfCHT1 as designed in the expression construct. The
trx-rPfCHT1 fusion protein and its enterokinase-cleaved
product rPfCHT1 had similar enzymatic activity. These
experimental results, in conjunction with the comparison
of the primary structures of PfCHT1 and PgCHT1 (Fig. 11),
10 strongly suggest that PfCHT1 is not produced as a
zymogen. This finding contrasts with previous
suggestions that P. gallinaceum ookinete-secreted
chitinase activity is synthesized as a zymogen that is
activated by ookinete protease(s) (Shahabuddin et al.
15 1993; Shahabuddin et al. 1995; Shahabuddin et al. 1996;
Shahabuddin 1998).

EXAMPLE XIII

Substrate Specificity of rPfCHT1. rPfCHT1 was found to
20 digest polymeric chitin efficiently in a nondenaturing
polyacrylamide activity gel into which glycol chitin had
been incorporated. TLC was used to further
characterizethe action of rPfCHT1 on native and
4MU-derivatized chitin oligosaccharide substrates (Figs.
25 14a-14c). rPfCHT1 had no hydrolytic action on GlcNAc$_2$ or
GlcNAc$_3$ but some activity on GlcNAc$_4$. rPfCHT1 had
markedly more activity against the longer native chitin
oligosaccharide substrates, GlcNAc$_5$ and GlcNAc$_6$ (Fig.
14a). rPfCHT1 did not cleave 4MU-GlcNAc or 4MU-GlcNAc$_2$
30 substrates, but cleaved the longer substrates (Fig. 14b).
4MU-GlcNAc$_3$ was hydrolyzed at only one glycosidic linkage,
yielding 4-MU and GlcNAc$_3$. Cleavage of 4MU-GlcNAc$_3$ was
rapid, about three times faster than from 4MU-GlcNAc$_4$, as
measured by microfluorimetry (Fig. 14c). The likely

reason for the slower release of 4MU from 4MU-GlcNAc$_4$ is
that the enzyme also cleaves two other glycosidic bonds,
yielding 4MU-GlcNAc and 4MU-GlcNAc$_2$, which cannot be
hydrolyzed (Fig. 14b). These data indicate that rPfCHT1

5 acts as an endochitinase, similar to P. gallinaceum
ookinete-secreted chitinases.

EXAMPLE XIV

pH Profile and Allosamidin Sensitivity of rPfCHT1.

10 pH-dependent activity profiles and allosamidin inhibitory
concentration curves for rPfCHT1 were determined by
microfluorimetry (Figs. 15a-15b). The sensitivity of
rPfCHT1 to allosamidin inhibition increases with rising
pH; no difference in the IC$_{50}$ curve is seen above pH 6.0.

15 The IC$_{50}$ of rPfCHT1 to allosamidin is 40 nM, less than
that of PgCHT2 (300 nM); both are distinctly different
from the IC$_{50}$ of rPgCHT1 (12 μM). The allosamidin
concentration (0.1-1.0 mM) sufficient to block oocyst
development in vivo (Shahabuddin et al. 1993) far exceeds

20 the IC$_{50}$ of PfCHT1 (40 nM) for allosamidin in vitro,
consistent with the hypothesis that PfCHT1 is involved in
allowing the ookinete to penetrate the PM.

EXAMPLE XV

25 **Molecular Modeling of PfCHT1.** PfCHT1 is predicted to
have an $(\alpha\beta)_8$ triose isomerase barrel structure typical of
family 18 chitinases (Terwisscha van Scheltinga et al.
1996). A majority of the active-site residues of PfCHT1
are common to either hevamine or Serratia marcescens

30 chitinase ChiA, for which crystal structures are
available (Terwisscha van Scheltinga et al. 1996;
Perrakis et al. 1994). The Plasmodium chitinases are
unique in that they have a Gly for Phe/Met
(hevamine/ChiA, respectively) change at a position (353

for PfCHT1, 405 for PgCHT1) that is highly conserved
among other family 18 chitinases. This position is in a
critical area at the base of the catalytic site (Fig. 16)
and may impart a unique structure. To explore further
5 the potential implication of this position as a site for
selective drug targeting, homology models were built for
PfCHT1, PgCHT1, and human chitotriosidase (Boot et al.
1995) (Fig. 16). Although the Gly for Phe/Met change
substantially enlarges the base of the catalytic pocket
10 in PgCHT1, a complementary Tyr^{309} in PfCHT1 on the β-7
strand compensates for the missing volume, resulting in
an almost perfect overlap of the catalytic pocket with
that of human chitotriosidase. In contrast, the I^{361}
change in the PgCHT1 β-7 strand does not fully compensate
15 for the Gly for Phe/Met change. The resulting unique
pocket distinguishes PgCHT1 from PfCHT1 and may explain
the differential sensitivity of PfCHT1 and PgCHT1 to
allosamidin. In the model, allosamidin does not appear
to contact Gly^{405} of PgCHT1 but does appear to contact
20 Tyr^{309} in PfCHT1.

EXAMPLE XVI

Plasmodium falciparum. The above Examples report that
PfCHT1 encodes an endochitinase, expressed by parasites
25 within the mosquito midgut, with a marked preference for
longer chitin oligosaccharide substrates, consistent with
the predicted biological function of this enzyme. This
substrate specificity is, to date, unique to Plasmodium
chitinases.

30 The data provides evidence that Plasmodium ookinetes
are likely to secrete products of more than one chitinase
gene. The pH profile of rPfCHT1 and its sensitivity to
allosamidin closely correspond to a second P. gallinaceum
ookinete-secreted chitinase activity that is

provisionally called PgCHT2 (see earlier Examples
relating to Plasmodium gallinaceum). The pH activity
profiles of both rPfCHT1 and PgCHT2 are shifted 0.5 pH
units toward the acid range compared with both

5 recombinant and native PgCHT1; rPfCHT1 and PgCHT2 also
become irreversibly inactivated at 0.5 pH units lower
than native and recombinant PgCHT1. rPfCHT1 has a
sensitivity to allosamidin much closer to that of PgCHT2
than to PgCHT1. PfCHT1 has other features in common with

10 PgCHT2: its size (molecular mass of secreted protein of
39 kDa vs. 35 kDa for PgCHT2; ookinete-secreted native
PgCHT1 has a molecular mass of 55 kDa) and the apparent
lack of a chitin-binding domain. It appears that PfCHT1
is the ortholog of PgCHT2.

15   As a target of blocking malaria parasite
transmission to mosquitoes, chitinase differs from
previously identified surface antigens of sexual stage
parasites in that it has a well characterized biochemical
activity at which nonimmunological interventions can be

20 aimed. In addition to the traditional transmission-
blocking vaccine approach, computational and structural
biology-driven rational drug design can be used to
identify chitinase-inhibitory drugs that block
transmission. Such chitinase inhibitors which are

25 nontoxic, inexpensive, highly potent, and bioavailable
with a long half-life in vivo can be administered widely
to human populations in endemic regions. There are
precedents for administering pharmacological compounds to
human populations in the water or food supplies:

30 fluoridation of water supplies, iodination of salt,
diethylcarbamazine in salt (Gelband 1994), and even
quinine in tonic water.

       Finally, Plasmodium chitinase can be used as a
target in generating transgenic mosquitoes (Besansky and

Collins 1992; Coates et al. 1998; Jasinskiene et al.
1998) that are refractory to ookinete invasion by
secreting a chitinase inhibitory peptide into the midgut.
An oligopeptide chitinase inhibitor selective for P.
5 falciparum chitinase can be obtained from screening
combinatorial phage display libraries as described above.
A synthetic gene encoding the peptide under the control
of a gut-specific, blood meal-inducible promoter such as
that of carboxypeptidase (Tellam et al. 1999) or late
10 trypsin could be used, in an appropriate construct, to
transform Anopheles mosquitoes to a refractoriness
phenotype.

Although preferred embodiments have been depicted
15 and described in detail herein, it will be apparent to
those skilled in the relevant art that various
modifications, additions, substitutions and the like can
be made without departing from the spirit of the
invention and these are therefore considered to be within
20 the scope of the invention as defined in the claims which
follow.

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